Etude de la composante microbiologique dans le cycle du mercure en zone polaire

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RESUME

Le mercure est un polluant global posant des problèmes de santé publique, il existe sous trois formes : la forme élémentaire gazeuse présente dans l'atmosphère, la forme divalente soluble présente en phase aqueuse et la forme organique toxique (méthylmercure). Il est émis par des sources naturelles et anthropiques, sous forme élémentaire, et est transporté sur de longues distances, sa concentration est donc homogène tout autour du globe.

Aux pôles, une chimie particulière amène à une oxydation du mercure de l'atmosphère qui provoque des dépôts de mercure divalent sur la neige, ces phénomènes sont appelés déplétions de mercure.

En parallèle les niveaux de mercure mesurés dans les écosystèmes polaires, spécialement en Arctique, sont élevés, sans que l'origine de ce mercure soit comprise.

Les micro organismes sont connus pour pouvoir réagir avec le mercure soit en le réduisant sous sa forme volatile, soit en le méthylant ou encore par des phénomènes d'adsorption. Mais ces phénomènes biologiques ont été peu étudiés en Arctique spécialement dans la neige.

Ce travail de thèse propose d'étudier la composante microbiologique dans le cycle du mercure en zone polaire afin de comprendre si elle joue un rôle dans l'entrée du mercure dans les écosystèmes polaires. Pour réaliser cette étude nous avons travaillé sur différents types d'échantillons.

Tout d'abord des micro organismes ont été isolés à partir de la neige arctique. Ils ont été identifiés et leur capacité à dégrader des acides organiques présents dans la neige a été testée. Ensuite leur comportement vis à vis de la température a été investigué afin de connaître leur température optimale de croissance.

La suite du travail a porté sur leur comportement vis à vis du mercure grâce à différentes manipulations comme la recherche de gènes de résistance, la mesure des Concentrations Minimales Inhibitrices et enfin par des manipulations d'interaction entre le mercure et le méthylmercure en quantité environnementales et les levures.

Une autre partie des recherches a porté sur des mesures de mercure dans du plancton échantillonné en arctique au printemps 2007, afin d'étudier si les dépôts de mercure sur la neige, survenu durant cette période, pouvaient avoir une influence sur les niveaux mesurés. Finalement, des mesures de mercure et

de méthylmercure ont également été effectuées dans des échantillons de biofilms récoltés en arctique et en antarctique.

Les résultats obtenus indiquent que la neige arctique renferme des micro organismes, 3 levures et 10 bactéries ont pu être isolés et identifiés. De par leur capacités de dégradation d'acides organiques et leur comportement face à la température, ces micro organismes semblent pouvoir se développer dans la neige au moment de la fonte.

Vis à vis du mercure, des gènes de résistance ont été mis en évidence chez 2 souches bactériennes indiquant que celles ci étaient capables de réduire la mercure divalent en mercure élémentaire. La tolérance des micro organismes au mercure, testée grâce à des mesures de concentrations minimales inhibitrices a montré que les souches supportaient des taux différents de mercure, les levures tolérant des concentrations plus élevées que les bactéries. Pour conclure ces travaux, les expériences d'interactions entre des levures et des espèces mercurielles ont montré que ces micro organismes pouvaient réduire le mercure divalent en mercure élémentaire et stocker le méthylmercure sans le déméthyler.

Finalement les mesures de mercure dans le plancton prélevés au printemps 2007 ont indiqué des taux très bas de mercure total qui ne semblaient pas varier selon les dépôts de mercure observés sur la neige.

Les mesures de mercure et méthylmercure dans les échantillons de biofilms arctiques et antarctiques ont donné des résultats proches des concentrations habituellement mesurées, indiquant que ce type de structure biologique pouvait stocker du mercure dont une partie sous forme de méthylmercure.

L'ensemble de ce travail a permis de montrer que la composante microbiologique était présente dans la neige, et pouvait avoir un rôle dans la chimie du mercure en zone polaire. Ce type d'approche à l'interface entre la chimie et de la biologie était une première au laboratoire de glaciologie, les résultats obtenus ouvrent des perspectives intéressantes pour la compréhension du cycle du mercure en zone polaire.

SUMMARY

Mercury is a global pollutant, which causes public health problems. It exists under three forms: the elemental one is a liquid with high vapour pressure, therefore present in the atmosphere, the divalent one present in aqueous form and the organic one (methylmercury) that is the more toxic between the mercury species.

Mercury is emitted by both natural and anthropogenic sources, under its elemental form it is transported all across the globe.

In polar areas, a particular chemistry leads to an oxidation of elemental mercury of the atmosphere in divalent mercury that is deposited onto snow; this phenomenon is called Atmospheric Mercury Depletion Events.

In parallel, mercury levels measured in arctic ecosystems are high; the origin of such contamination is not well understood.

Microorganisms are known to be able to react with mercury by reduction, methylation or by adsorption. But these biological phenomenons were not studied yet in Arctic especially in snow.

This PhD propose to study the microbiological component in the mercury cycle in polar area, in order to understand if micro organisms have a role to play in the entry of mercury in the ecosystems. For this work some different kind of samples were studied.

First microorganisms were isolated from Arctic snow. They were identified and their ability to degrade organic acids from snow was investigated. Then their behaviour toward temperature was studied to determine their optimal growth temperature.

To continue this work, we focused on the behaviour of microorganisms with mercury. Some resistance genes against the metal were searched in bacteria and minimal inhibition concentrations for mercury were measured in both yeast and bacteria. The behaviour of yeast with environmental amount of mercury and methylmercury was also followed.

Another part of the work deals with total mercury measurements in plankton sampled during a field campaign in spring 2007 in order to see if mercury deposition has an impact on mercury content in plankton. Finally mercury and methylmercury measurements were performed in biofilms samples originated from Arctic and Antarctica.

The results obtained indicate that snow contains microorganisms. Ten bacteria and three yeasts were isolated and identified. They were able to degrade organic acids from snow and for certain strain, to tolerate low temperature. This shows that microorganisms could live in snow especially during spring snowmelt.

Concerning mercury, two bacteria possess mercury resistance genes, indicating they were able to reduce divalent mercury to elemental mercury.

The tolerance of microorganisms to mercury, tested by measuring minimum inhibition concentration, indicates that the strains could support different mercury levels, and that yeast tolerates higher mercury levels than bacteria. To conclude this work some interaction experiment between mercury species and yeast shows they could reduce divalent mercury to elemental mercury and store methylmercury without demethylate it.

Finally mercury measurements in plankton give low mercury values, which did not seem to be influenced by atmospheric mercury deposition.

The mercury and methylmercury measurements performed in biofilms samples give values close to the ones already measured in such biological samples. This confirms that this kind of biological structure could concentrate mercury with a proportion of methylmercury.

All this work permits to demonstrate that the microbiological component exists in the snow, and could play a role in mercury chemistry in polar area. This kind of research between chemistry and biology was one of the first one in this laboratory and the results obtained give interesting perspectives for understanding of the mercury cycle in polar area.

Chapter 1 : Presentation of the manuscript

This manuscript presents the work done during my PhD. As I worked in co-tutelle between the Laboratoire de Glaciologie et Géophysique de l'Environnement (L.G.G.E., Université Joseph Fourier, Grenoble, France) and the Dipartimento di Scienze Ambientali (University Ca'Foscari, Venice Italy), this manuscript is written in English and French. It is divided in 13 chapters, with this one dedicated to the presentation of the manuscript.

The chapter 2 presents a bibliographic study focused on mercury in Arctic, polluted Arctic ecosystems and micro-organisms. It poses the problematic of my work and explains why it was important to investigate the microbial content in snow in Arctic. This chapter is written in French with a summary in English.

The chapter 3 presents the different methods used during the PhD. It contains descriptions of the microbiological methods and also details of the different mercury measurements methods. This chapter is written in English.

The chapters 4 to 11 present the different results obtained during the PhD. All these chapters are written in English and the discussion on the data obtained are integrated in each of them.

Here bellow is reported the general structure of the results:

- Chapter 4 : Micro organisms isolated from snow
- Chapter 5: Growth characteristics of micro organisms isolated from snow
- Chapter 6: Mercury inhibition concentration for micro organisms isolated from snow
- Chapter 7: Resistance genes in Arctic bacteria
- Chapter 8: Behaviour of yeast with inorganic mercury
- Chapter 9 : Behaviour of yeast with methylmercury
- Chapter 10: Total mercury measurements in planktonic samples during a field campaign
- Chapter 11: Total and methyl mercury measurements in polar non marine biofilms

The chapter 12 presents the conclusions and the perspectives of the PhD written in both French and English and the chapter 13 presents the literature cited in the manuscript.

Chapitre 2 : Synthèse bibliographique

1. Le mercure

1.1 Historique et utilisation

Pour beaucoup d'entre nous lorsque l'on parle de mercure on pense immédiatement aux thermomètres à mercure qu'il était interdit de toucher lorsqu'ils étaient brisés. Nous restions alors fascinés par cet étrange élément, ce métal liquide, qui se présentait sous forme de billes et qui brillait comme de l'argent.

Figure 2.1 : Gouttes de mercure élémentaire sous forme liquide

Le mercure est connu depuis l'antiquité, de part son aspect liquide et brillant il était parfois appelé « vif-argent », ce qui en latin se traduisait par *Hydrargyrum*, c'est de ce nom que provient le symbole chimique du mercure : Hg.

Le mercure a de nombreuses utilités, sous forme élémentaire, de par ses capacités d'amalgamation il peut être utilisé pour extraire l'or ou l'argent.

Ses propriétés physiques particulières (densité et coefficient de dilatation thermique élevés) lui ont values d'être utilisé dans les thermomètres, les

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Figure 1

baromètres ou les manomètres. On le connaît également pour son utilisation dans les amalgames dentaires.

Sous sa forme divalente, il est biocide et antiseptique, il peut donc être utilisé comme tel (mercurochrome, utilisation comme pesticide par exemple).

1.2 Les différentes formes du mercure

Le mercure est le 80ème élément chimique de la classification périodique, il est l'unique métal liquide à température et pression ambiante, et il existe sous trois degrés d'oxydation : 0, +I et +II.

Dans l'atmosphère, il est présent à 95% sous sa forme volatile non toxique : Hg^0 (Slemr et al. 1985) ; (Munthe 1994).

Sous cette forme élémentaire (Hg^0), il est peu soluble et volatil, une goutte de mercure liquide laissée à l'air libre va donc vite disparaître pour se vaporiser dans l'atmosphère.

Grâce à un temps de vie dans l'atmosphère d'environ 1 an, sa concentration atmosphérique est relativement homogène tout autour du globe (Poissant et al. 2002).

Les concentrations habituellement mesurées étant comprises entre 1 et 4 ng/m^3 d'air (Lindqvist and Rodhe 1985; Lindberg and Stratton 1998), le niveau de fond moyen dans l'hémisphère nord est de 1,7 ng d'Hg^0/m^3 d'air (Slemr et al. 2003).

Cette forme élémentaire peut être oxydée en mercure divalent Hg^{2+}. Dans l'atmosphère, cette oxydation se fait grâce à des radicaux O_3, OH , Cl ou Br (Sommar et al. 2001) ; (Ariya et al. 2002).

Les principales espèces divalentes inorganiques formées sont alors $HgCl_2$, $Hg(OH)_2$, HgO ou $HgBr_2$.

Ainsi oxydé il devient alors plus soluble et de part cette solubilité il est sujet au lessivage par les précipitations, ce qui entraîne des dépôts humides (pluies et neige) qui sont l'une des principales sources des apports de mercure dans l'environnement (Hudson et al. 1995).

C'est donc sous cette forme divalente qu'on le retrouve dans les eaux atmosphériques (Lin and Pehkonen 1999).

Le mercure divalent, une fois lessivé rejoint alors les écosystèmes aquatiques où il peut être méthylé principalement par action bactérienne (Pongratz and Heumann 1998).

1.3 Origines du mercure

1.3.1 Naturelles

Le mercure est un élément naturel de la croûte terrestre qui a une abondance moyenne de 0,05 mg/kg.

C'est également un produit résultant de l'activité volcanique terrestre et sous marine. Les émissions naturelles sont estimées entre 2000 et 3000 T/an (Lindqvist et al 1985).

Plusieurs dizaines de minerais peuvent renfermer naturellement du mercure mais les plus utilisés pour extraire ce métal sont à base de cinabre HgS.

En effet cette roche peut contenir jusqu'à 1% de mercure, certains gisements pouvant même renfermer jusqu'à 14 % de ce métal.

Enfin les dernières sources naturelles de mercure sont l'évaporation à partir des sols et des eaux de surface ainsi que les feux de forêts (UNEP 2002).

Il semblerait que les rejets naturels de mercure représentent moins de 50% des rejets totaux (Munthe et al. 2001).

1.3.2 Anthropiques

Car une bonne partie du mercure présent dans l'atmosphère a une origine anthropique soit par une utilisation intentionnelle dans des produits ou des procédés, soit par rejet lors de combustion d'énergies fossiles, d'industries chimiques, d'incinération de déchets, ou par les gaz d'échappement.

Les rejets d'origine anthropique sont estimés à 4000 T/an (Pirrone et al. 1996).

Son utilisation intensive ainsi que les émissions dans l'atmosphère depuis la révolution industrielle ont répandu d'importantes quantités de ce polluant dans l'environnement en plus des apports naturels.

Cette introduction massive de mercure dans l'atmosphère pose des questions environnementales et de santé publique, car le mercure peut être très toxique pour les êtres vivants.

1.4 Toxicité des formes de mercure

Sous forme élémentaire liquide, Hg^0, le mercure n'est pas très dangereux lorsqu'il est en contact avec la peau, mais l'inhalation de ses vapeurs peut provoquer des irritations des voies respiratoires, principalement à cause de son oxydation en Hg^{2+} (Gochfeld 2003).

Sous forme divalente, l'effet le plus prononcé de l'exposition chronique (ingestion de Hg^{2+} ou d'espèces organométalliques) est neurologique et psychiatrique.

Sous forme organique (méthylmercure) il est plus lipophile que Hg^{2+}, il attaque le système nerveux central en réagissant avec les groupements thiols des protéines, enzymes et autres macromolécules, ce qui perturbe leurs fonctions.

Il peut également passer à travers les membranes, la barrière hémato-encéphalique et le placenta. Les autres dommages dont il est responsable concernent les reins, et le système immunitaire (Gochfeld 2003).

La contamination principale des humains et autres êtres vivants se fait par la chaîne alimentaire car le méthylmercure est bioaccumulable, lorsqu'il est ingéré par des êtres vivants (ingestion d'eau ou de proies contaminées), ils ne peuvent pas l'éliminer (Atwell et al. 1998).

Comme il n'est pas éliminé, la contamination en mercure provoque un phénomène de bio-amplification le long de la chaîne alimentaire. Les concentrations internes en mercure augmentent avec le temps d'exposition ainsi qu'avec le rang occupé dans la chaîne alimentaire. Les prédateurs situés en haut de chaîne étant donc plus atteints que les proies situées au bas de la chaîne alimentaire. (Japan Public Health, 2001)

On peut calculer un facteur de bioconcentration (FBC) entre la concentration dans la colonne d'eau et celle dans les poissons, dans l'arctique canadien le FBC est de $\sim 10^6$ (Muir et al. 1999).

Comme il est capable de traverser la barrière hémato-encéphalique le méthylmercure provoque à forte dose des troubles neurologiques graves et irréversibles, la contamination la plus tristement célèbre étant celle de la baie de Minamata au Japon en 1956 (Osame and Takizawa 2001)

A l'époque une usine d'acétaldéhyde qui utilisait du mercure comme catalyseur rejetait ses déchets mercuriels dans la baie. L'écosystème était donc très pollué et les poissons renfermaient des taux de méthylmercure très élevés.

Les habitants, qui se nourrissaient essentiellement de poissons furent donc fortement empoisonnés. Ils ont alors développé des symptômes neurologiques graves. On a recensé plus de 2000 cas de la « maladie de Minamata », et depuis 1956, 1000 décès ont été enregistrés. Les manifestations les plus dramatiques (retard de développement cérébral et moteur) étant observées chez des enfants nés de mères exposées pendant leur grossesse (Gochfeld 2003).

Une autre contamination médiatisée s'est produite en Irak en 1971, 459 morts et plus de 6000 hospitalisations ont été répertoriés à la suite de la consommation par erreur de semences traitées par des dérivés mercuriels afin de leur garantir une meilleure conservation (Bakir et al. 1973).

De par sa toxicité, il existe des recommandations sanitaires concernant les taux de mercure dans les denrées alimentaires (principalement les poissons), par exemple l'US-EPA (United States Environmental Protection Agency) recommande de ne pas consommer plus de 0,1 µg de mercure/kg de poids corporel/jour.

2. Le cycle du mercure dans l'environnement

2.1 Cycle général

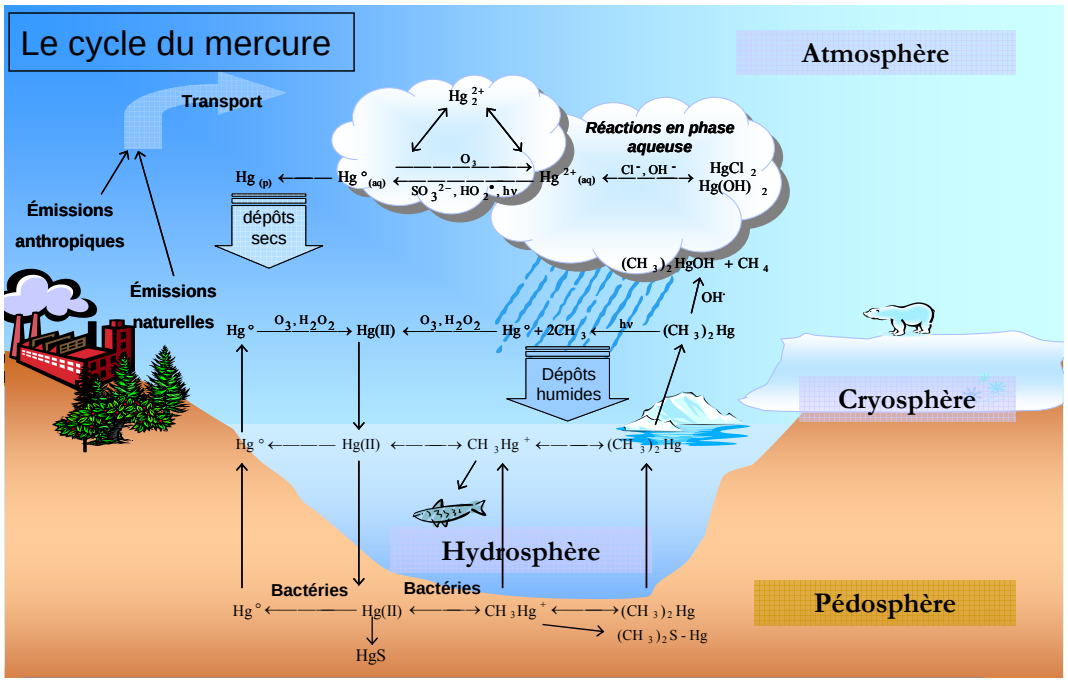

Figure 2.2 : Schéma général du cycle du mercure

La Figure 2.2 présente le cycle général du mercure. On observe que le mercure est émis par des sources naturelles et anthropiques dans l'atmosphère sous forme élémentaire Hg^0.

Il peut ensuite être oxydé en mercure divalent au niveau des nuages par l'ozone et certains radicaux.

Une fois oxydé, il peut alors former des complexes avec certains ions présents dans les nuages tels que les chlorures ou les hydroxydes, ce qui amène à la formation de complexes comme $HgCl_2$ et $Hg(OH)_2$ qui sont alors lessivables par les précipitations et peuvent donc rejoindre la biosphère. On appelle ce type de dépôts de mercure les dépôts humides.

Le mercure atmosphérique peut également s'associer à des particules pour former des dépôts secs, ce mercure est appelé mercure particulaire ($Hg_{(p)}$).

Une fois déposé dans la biosphère le mercure divalent peut subir de nombreuses réactions d'oxydation ou de réduction à différentes interfaces (eau-air, sol-eau par exemple).

Il peut également se déposer au fond des océans au niveau des sédiments. C'est dans ce compartiment qu'il peut être méthylé sous l'action de bactéries sulfato-réductrices. Cette méthylation se fait en stricte anaérobiose, c'est à dire sans oxygène, et en présence de sulfates (Compeau and Bartha 1985),(King et al. 2002),(Pongratz and Heumann 1998).

2.2 Une chimie particulière aux Pôles

2.2.1. Les Déplétions de mercure

Nous avons donc vu les différentes formes de mercure et les différentes réactions auxquelles cet élément est soumis dans l'atmosphère, sous nos latitudes.

Or selon la latitude les réactions de chimie atmosphérique qu'il subit ne sont pas les mêmes.

En 1998, un phénomène chimique totalement nouveau a été découvert en Arctique par W. H. Schroeder (Schroeder et al. 1998).

Au printemps, et dans certaines conditions de températures et d'irradiation, on a pu observer que tout le mercure élémentaire gazeux contenu dans l'atmosphère disparaissait soudainement.

Ce phénomène a été appelé « Déplétions Atmosphériques de Mercure » soit « Atmospheric Mercury Depletion Event » ou A.M.D.E. (Ebinghaus et al. 2004).

En un temps très rapide on pouvait voir la concentration de Hg^0 atmosphérique passer des quelques ng/m^3 habituels à des concentrations quasiment nulles comme le montre la figure 2.3.

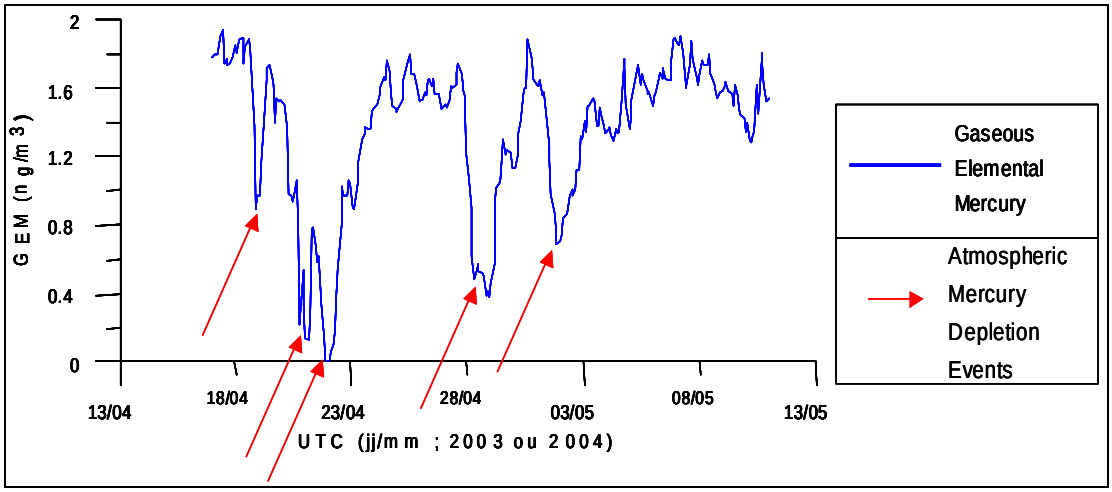

Figure 2.3 : Enregistrements des concentrations de mercure élémentaire gazeux dans l'atmosphère de Ny-Ålesund, Svalbard, Norvège au printemps 2003. Les flèches rouges indiquent les événements de déplétion de mercure.

La question du devenir du mercure s'est donc posée.

Nous avons vu précédemment que le mercure avait une concentration constante dans l'atmosphère tout autour du globe, y compris au dessus des pôles de l'ordre de 1,5-1,7 ng/m^3 et qu'excepté lors des précipitations il était plutôt peu réactif sous nos latitudes.

Pour comprendre ce qui arrive au mercure il faut se pencher de plus près sur la chimie atmosphérique des régions polaires arctiques. Car des observations ont prouvées que les AMDE étaient en phase avec d'autres phénomènes particuliers : Les Low Ozone Events et les bromine explosion.

2.2.2 Les Low Ozone Events

Les Low Ozone Events (LOE) ont été observés pour la première fois en 1986 par Oltmans et Komhyr à Barrow en Alaska. Ils ont pu constater qu'au printemps polaire, les concentrations en ozone dans la basse troposphère pouvaient, elles aussi, chuter jusque sous les limites de détection et ceci en quelques heures.

Diverses études ont montrées que le radical Br avait un rôle très important dans le mécanisme des LOE (Barrie et al. 1988; Finlayson-Pitts and Livingston 1990).

En effet, dans l'atmosphère arctique, Br peut être oxydé par l'ozone ce qui mène à la formation du radical BrO par la réaction $Br_{(g)} + O_{3(g)} \rightarrow BrO_{(g)}$.

Ce phénomène est appelé destruction de l'ozone.

Or le radical $BrO_{(g)}$, une fois formé est capable d'attaquer certains composés de l'air selon différentes réactions présentées ci après (Tuckermann et al. 1997):

$$BrO_{(g)} + XO_{(g)} \rightarrow Br_{(g)} + X_{(g)} + O_{2(g)}$$

avec X= Br, Cl ou I

ou

$$BrO_{(g)} + HO_{2(g)} \rightarrow HOBr_{(g)} + O_2$$

puis

$$HOBr_{(g)} + h\nu \rightarrow Br_{(g)} + OH_{(g)}$$

Dans tous les cas, des destructions aussi rapides de l'ozone nécessitent de fortes quantités de Br, la question de l'origine de ce brome a donc été étudiée.

Très rapidement le rôle des surfaces actives, comme la neige, les aérosols ou les frost flowers, a été mis en évidence, car ces surfaces contiennent de fortes quantités d'ions Br^- et d'oxydants comme HOBr.

Or au printemps polaire, sous l'effet des UV, ces ions Br- et ces oxydants HOBr sont transformés en $Br_{(g)}$ et $BrO_{(g)}$ puis libérés dans l'atmosphère.

Ce sont les radicaux $Br_{(g)}$ et $BrO_{(g)}$ qui peuvent ensuite oxyder le mercure élémentaire en mercure divalent (Lindberg et al. 2002).

Les réactions d'oxydation du mercure atmosphérique étant les suivantes, les espèces mercurielles se déposant sur la neige sont indiquées en rouge :

$$Br_{(g)} + Hg° \rightarrow HgBr$$

$$BrO_{(g)} + Hg° \rightarrow HgO + Br$$

ou

$$BrO_{(g)} + Hg° \rightarrow HgBr + O$$

ou

$BrO_{(g)} + Hg° + X$ qui donnera selon X (X= Br, Cl , OH, O_2 ou I) :

$Hg(OH)_2$, $HgBr_2$, HgBrCl, HgBrI, HgBrOH et $HgCl_2$

La Figure 2.4 résume le mécanisme des AMDE dans sa globalité.

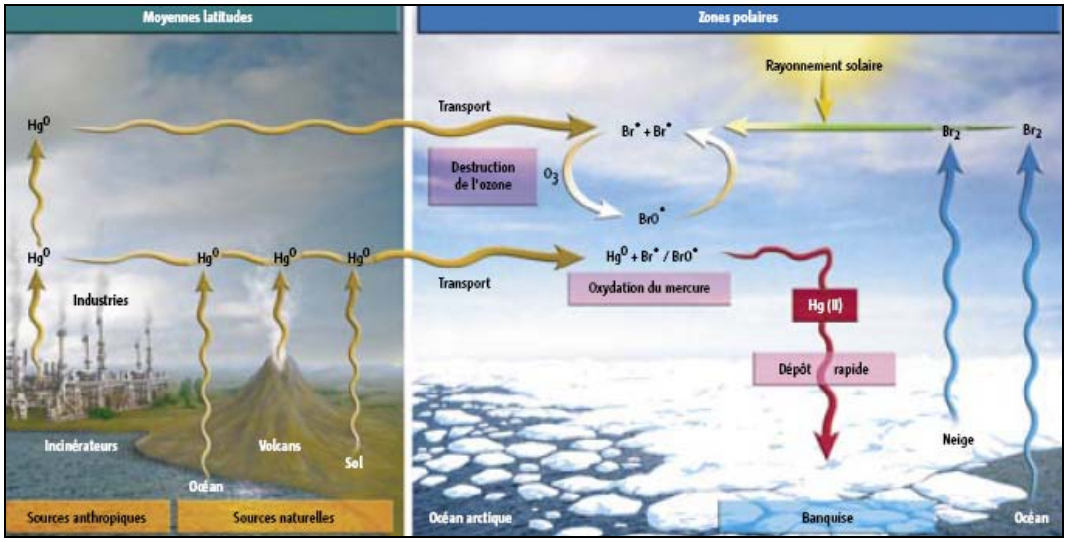

Figure 2.4 : Schématisation des mécanismes aboutissant aux pluies de mercure en zone polaire (Source : La Recherche, décembre 2004)

Lors de ces AMDE il y a donc un dépôt de mercure sous forme d'espèces mercurielles sur la surface de la neige.

Les concentrations de mercure enregistrées (toutes espèces confondues) dans la neige sont de l'ordre de plusieurs dizaines à plusieurs centaines de nanogrammes par litre de neige fondue une fois déposé (Poulain et al. 2004).

Mais on ne connaît pas exactement son devenir, il semblerait qu'une partie importante de ce mercure soit volatilisée vers l'atmosphère (Ferrari et al. 2004).

Le reste pourrait être transféré vers les écosystèmes à travers les eaux de fontes (Loseto et al. 2004).

Le manteau neigeux semble donc jouer un rôle clé dans la chimie du mercure pendant le printemps polaire. De plus, lors de la fonte, il pourrait également jouer un rôle dans le transfert du mercure déposé vers les écosystèmes.

Car les écosystèmes arctiques sont contaminés par le mercure, dans la partie suivante nous allons présenter ces écosystèmes ainsi que la contamination dont ils sont victimes.

3. Les écosystèmes en arctique

3.1 Caractéristiques biologiques de la chaîne alimentaire arctique

Le figure 2.5 présente un schéma de la chaîne alimentaire arctique, elle nous indique que le phytoplancton est consommé par le zooplancton, ainsi que par les poissons « filtreurs » (comme la baleine par exemple). Le zoo plancton est consommé par des poissons tels que la morue arctique, l'omble, ou le flétan.

Les poissons sont eux même consommés par les phoques barbus et annelés dont le principal prédateur est l'ours polaire.

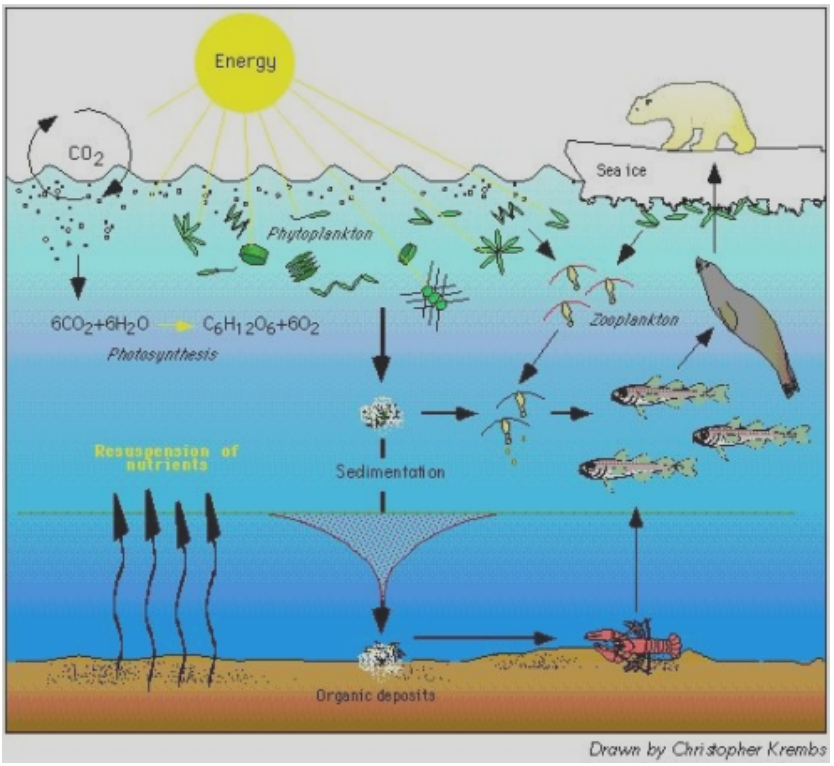

Figure 2.6 : Représentation de la chaîne alimentaire arctique

En arctique, à cause des faibles apports en nutriment, du climat froid et de la faible durée de la saison de croissance la productivité est basse.

Les dépôts atmosphériques de nutriments et de matrices organiques, les remontées d'eaux riches en nutriments venues du sud et les apports riverains de nutriments sont tous importants dans la productivité des écosystèmes marins.

Cette productivité est très cyclique, à cause des fluctuations extrêmes du niveau lumineux, des apports en nutriments et de la température.

Les animaux et les plantes se sont adaptés à ces fluctuations de différentes manières. Certains migrent, d'autres se dispersent vers des habitats plus cléments. C'est pourquoi les niveaux de contamination en polluant ne sont pas toujours en rapport avec les dépôts locaux (Morel et al, 1998).

3.2 Contamination des écosystèmes arctiques.

Des études concernant la contamination en mercure des biotopes arctiques existent, mais elles concernent principalement le nord du Canada.

En effet, dans cette région, la recherche systématique d'une contamination est effectuée en raison des taux de mercure élevés habituellement retrouvés dans les poissons. Car ces taux dépassent parfois ceux préconisés par le « Health Canada Consumer Guideline » qui sont, pour le poisson, de 0,5 µg de mercure total/g poids frais.

Ces études indiquent que la chaîne alimentaire est contaminée par le mercure avec des taux variables selon le maillon considéré.

Notons que la distribution et les mécanismes de transfert de ces composés entre les différents compartiments et dans la chaîne alimentaire marine sont très peu compris.

Selon Atwell et al, 1998, les niveaux de mercure total dans les muscles des invertébrés du nord canadien, varient de 0,03 à 0,15 µg/g de poids sec selon les espèces.

Chez les poissons les niveaux sont de l'ordre de 0,2 µg/g de poids sec et chez les oiseaux on retrouve des niveaux variant entre 0,33 et 1,86 µg/g de poids sec.

Dans le zooplancton principalement composé de copépodes (*Calanus hyperboreus*, *Themisto libellula* et *Mysis oculata*) les niveaux varient de 0,006 à 0,025 µg/g de poids secs.

Une autre étude très complète concernant l'arctique canadien et a été réalisée par Muir et al en 1999.

Les niveaux de contamination indiqués ci après proviennent de cette étude.

Chez les moules (*Mytilus edulis*) les concentrations moyennes de mercure total sont relativement basses à 0,01 à 0,03 µg/g poids frais.

Chez les mollusques on a mesuré de 0,011 à 0,023 µg/g, et chez les crustacés, les valeurs moyennes sont comprises entre 0,04 et 0,88 µg/g.

Le niveaux de mercure total dans le foie des phoques de l'Ouest de l'Arctique est à 32,9 µg/g de poids frais, dans les autres régions de l'arctique il est 4 fois inférieur de l'ordre de 8,34 µg/g de poids frais.

Les concentrations moyennes de mercure dans les muscles des bélugas et des narvals sont respectivement de 1,34 et 0,81µg/g de poids frais.

Les ours polaires sont les principaux mammifères prédateurs du haut de la chaîne alimentaire arctique. Ils sont largement distribués dans les régions du

cercle polaire arctique et sub-arctique et leur régime alimentaire est principalement constitué de phoques.

En ajustant sur l'age des individus on trouve des niveaux de mercure dans le foie variant entre 20 et 200 µg/g de poids sec selon la localisation des individus.

Dans l'arctique canadien 92 % des phoques échantillonnés ont des taux de méthylmercure dans les muscles supérieurs à la norme tolérée de 0.5 µg/g de poids frais.

3.3 Mercure et santé publique

La contamination par le mercure pose des problèmes de santé publique pour les populations vivant près des pôles et ayant un régime alimentaire à base de poissons (groenlandais, inuits) spécialement pour les groupes à risques comme les femmes enceintes ou allaitantes et les jeunes enfants.

En effet les niveaux de méthylmercure mesurés chez ces populations sont bien au dessus de ceux retrouvés chez des individus vivant sous d'autres latitudes (Hansen, 2000).

Des recommandations concernant la consommation de poissons ont donc été mises en place pour limiter l'exposition au méthylmercure.

L'US-EPA a définit une dose de référence (RFd) correspondant à la quantité de mercure qu'un individu (enfant inclus) peut consommer tous les jours durant toute sa vie sans effets nocifs. Cette RFd est de 0,1 µg/kg de poids corporel/jour.

Dans toute chaîne alimentaire, les déchets, les matières organiques issues de la précipitation ou de la matière organique morte, sont pris en charge par les microorganismes.

Les nutriments qui en résultent contribuent à la production primaire. Cette voie est peu importante en terme de masse totale de carbone mais peut être importante dans le transfert des contaminants dans la chaîne alimentaire comme c'est le cas pour le mercure.

C'est pourquoi nous nous sommes intéressés aux microorganismes. Dans la partie suivante nous allons présenter un rappel du rôle des microorganismes dans l'environnement ainsi qu'un point sur les connaissances concernant les

microorganismes et les milieux froids. Nous terminerons ensuite par décrire leur comportement en présence de mercure.

4. Les micro-organismes

4.1 Rôle des micro-organismes dans l'environnement

Dans l'environnement, les microorganismes procaryotes ne participent pas directement au réseau trophique, mais ils jouent un rôle essentiel dans les écosystèmes.

En effet, ce sont des décomposeurs de la matière organique morte, une fois décomposée, elle est remise à disposition des producteurs primaires sous forme minérale (nitrates, sulfates, phosphates). Les micro-organismes permettent donc l'injection de nutriments dans la pyramide trophique.

Leur remarquable diversité de métabolismes les rends ubiquistes: il peuvent être thermophiles comme dans les sources chaudes (fumeurs noirs des fonds marins par exemple), halophiles dans les milieux très salins (mer morte, marais salants), ou tolérer des fortes doses de polluants comme dans les zones contaminées en métaux lourds, PCB et autres substances nocives.

4.2 Les micro-organismes des milieux froids

Les micro-organismes se retrouvent également dans les milieux glacés des pôles et des hautes altitudes.

Dans ces milieux, ils sont pour la plupart définis comme psychrophiles (Morita, 1975) ou au moins psychrotolérants (Junge et al, 2002).

La plupart forment des colonies de colorations jaunes ou oranges, témoignant d'adaptations physiologiques visibles au niveau pigmentaire (Skidmore et al, 2000).

Les études microbiologiques de ces zones extrêmes ont présenté un intérêt croissant ces dernières années, le challenge étant de connaître et de comprendre ces écosystèmes particuliers ainsi que les mécanismes d'adaptation au froid mis en place par les microorganismes.

De plus ces recherches laissaient entrevoir des applications intéressantes comme la dégradation de déchets à basse température, la fabrication et la

conservation d'aliments, ou l'utilisation industrielle d'enzymes travaillant à basse température (Margesin et al, 2003).

Des microorganismes ont donc été isolés à partir de différents milieux extrêmes comme

- la glace de mer
- les glaciers
- les neiges des pôles

Dans la glace de mer, on peut retrouver des virus, des bactéries, des algues, des protistes, qui vivent le plus souvent dans les réseaux de saumure ces réseaux étant connus pour renfermer une importante production biologique (Helmke and Weyland 1995) qui se présente majoritairement sous forme de biofilms qui s'accrochent à la glace grâce à des exopolymères (Krembs et al, 2001).

Au niveau bactérien, les études montrent une densité totale de l'ordre de 5.10^4 cellules/ml dans la glace propre, c'est-à-dire sans bande d'algues, dont 2 à 27% sont actives (Junge et al, 2002).

Les cultivables représentent une part importante de la population totale, par rapport aux autres milieux habituellement décrits soit 4 à 27%.

Les groupes bactériens les plus communément retrouvés sont, par ordre d'importance : γ-Protéobactéries, α-Protéobactéries, Cytophaga-Flavobacterium-Bacteroides (CFB) et en très faible proportion (<5%) : β-Protéobactéries et Actinobactéries (Brinkmeyer et al, 2003).

Enfin, les études récentes et poussées de la glace de mer ont permis de décrire de nouveaux genres bactériens et de nouvelles espèces bactériennes (Bowman et al, 1997), (Junge et al, 2002).

Des populations bactériennes ont été retrouvées dans les glaciers des Alpes (Sharp et al, 1999) et Tibet (Christner et al, 2003) (Zhang et al, 2002), lors d'études axées principalement sur la viabilité des microorganismes enfermés depuis de longues périodes dans la glace. Skidmore et al (2000) ont pu observer le développement de plus de 10^3 cellules/ml, toutes psychrophiles, après mise en culture d'échantillons de glace de glacier à 4°C (profondeur et âge de la glace non-précisés).

Les genres communément isolés sont essentiellement de α et de β-Proteobacterie (genres *Pseudomonas* et *Acinetobacter*), d'Actinobacterie, et de Gram-positifs à faible G+C% (Christner et al. 2003).

D'autres microorganismes comme des levures ont également été isolés à partir de neige de glacier arctique (Butinar et al, 2007) ainsi que des champignons (Gunde-Cimerman et al, 2003).

En ce qui concerne les pôles il existe des études plutôt axées sur le pôle Sud (Brinkmeyer et al. 2003) qui montrent que les genres bactériens isolés de la glace polaire sont majoritairement des souches appartenant aux alpha et gamma protéobactéries et au groupe des *Cytophaga-Flavobacterium.*

D'autres recherches ont montrées la présence des cyanobactéries vivants sous formes de biofilms (Jungblut et al. 2005).

Finalement il a été démontré que dans des conditions extrêmes les microorganismes présents avaient une activité métabolique (Carpenter et al. 2000).

Malgré l'intérêt croissant pour ce type de milieu, peu d'études sur les microorganismes de la neige arctique existent.

La composante microbiologique de la neige spécialement en milieu arctique, siège des AMDE, est donc à explorer.

4.3 Les micro-organismes et le mercure

4.3.1 Les bactéries résistantes

Dans les environnements contaminés par le mercure, certaines bactéries ont développé un mécanisme de résistance contre ce polluant.

Cette résistance est basée sur la synthèse de différentes protéines qui prennent en charge le mercure divalent pour le faire pénétrer dans la cellule et le réduire en mercure élémentaire. Le mercure élémentaire peut ensuite se volatiliser et regagner l'atmosphère. Ce système de résistance est inductible et il est gouverné par un opéron : l'opéron *mer.*

La Figure 2.5 représente la structure de l'opéron ainsi qu'une schématisation du mécanisme de résistance.

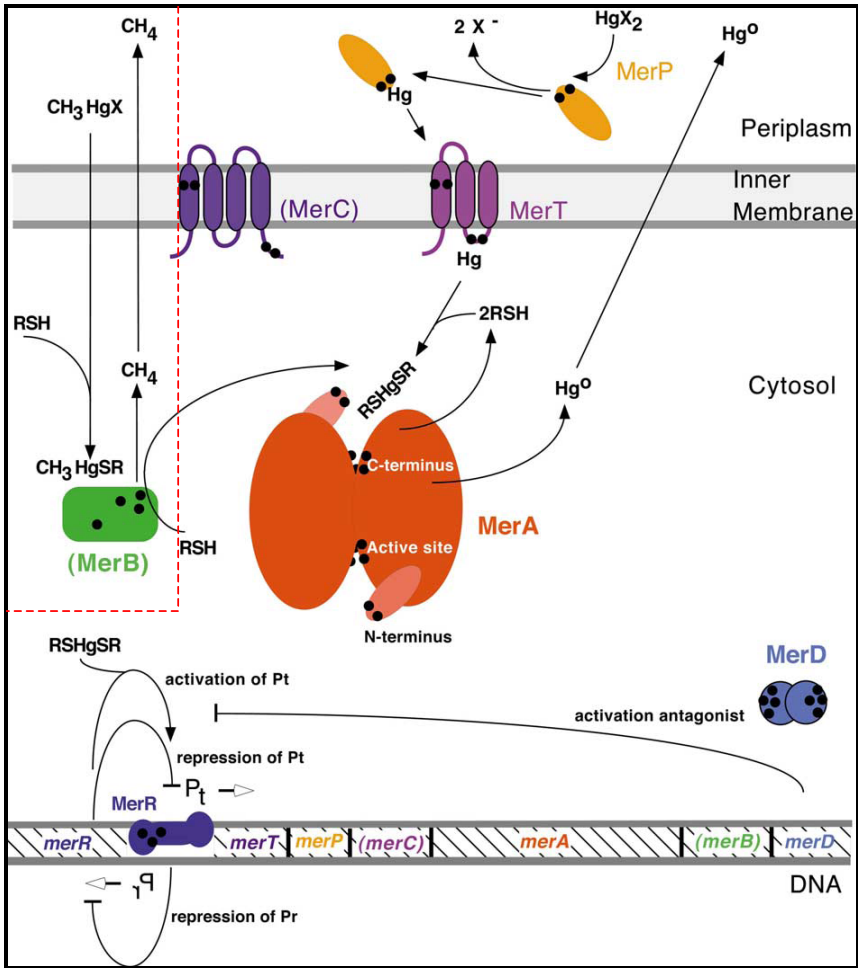

Figure 2.5: Organisation et fonctionnement de l'opéron mer chez les bactérie gram négatif. Le symbole ● représente les résidus de cystéine pouvant lier le mercure. RSH correspond à une molécule de faible poids moléculaire comme le glutathion (Barkay et al, 2003) Le mécanisme séparé par des pointillés rouges sera présenté dans la partie 4.3.2.

Cet opéron est composé de plusieurs gènes codant pour des protéines qui ont chacune un rôle spécifique dans la résistance au mercure.

Les principales protéines impliquées dans la résistance au mercure sont :

- MerT : protéine de transport du mercure de l'extérieur vers l'intérieur la cellule, elle est enchâssée dans la membrane cytoplasmique
- MerP : protéine de transport chargée d'acheminer le mercure à MerT dans le périplasme

- MerC : autre protéine de transport transmembranaire pas toujours présente, elle semble jouer un rôle d'aide pour MerT dans des environnements très contaminés en mercure
- MerA : c'est la « mercuric reductase » c'est à dire l'enzyme catalysant la réaction de réduction de Hg^{2+} en Hg^0
- MerR : répresseur de l'opéron
- MerD : antogoniste de MerR
- MerB : enzyme de dé-méthylation cf 4.3.2

L'activation de cet opéron se fait par fixation d'une molécule de mercure sur une partie du répresseur MerR, riche en cystéine, ce qui lève la répression sur l'opérateur MerO et initie la transcription du gène et donc la synthèse des protéines. En effet dans un environnement contenant de fort taux de mercure une quantité infime de métal peut passer par diffusion et activer la transcription des gènes adéquats.

Une fois activé le système de résistance prend en charge le mercure divalent, il se lie tout d'abord à la protéine MerP qui l'amène au niveau de la membrane cytoplasmique afin de le transmettre à MerT.

MerT lui fait alors traverser cette membrane plasmique et le présente à la réductase MerA qui le réduit en Hg^0.

Ce mécanisme se met en place très rapidement, il faut quelques minutes en présence de mercure pour qu'une bactérie résistante induise la synthèse des protéines adéquates (Noël-Georis et al, 2003).

Notons que tout au long de ce processus les cystéines jouent un rôle très important de part leur affinité pour le mercure.

4.3.2 Les bactéries dé-méthylantes

La partie gauche de la figure 2.5 entourée de pointillés rouges présente un mécanisme que l'on retrouve chez certaines bactéries résistantes. Ce mécanisme leur permet de dé-méthyler le méthylmercure. Il existe deux types de dé-méthylation : la dé-méthylation par réduction et la dé-méthylation oxydative.

La dé-méthylation par réduction se produit, en présence de fortes concentrations de méthylmercure en environnement aérobie. Cette réaction fait intervenir le système *mer* par l'intermédiaire de l'enzyme MerB, elle

s'accompagne de la libération d'un méthane qui est re-volatilisé vers l'atmosphère. Le mercure divalent issu de la dé-méthylation rejoint ensuite MerA qui le réduit en mercure élémentaire.

La dé-méthylation oxydative se produit en anaérobiose, en présence de basses concentrations de méthylmercure et la dégradation du méthylmercure entraîne la formation de CO_2 et d'une petite quantité de méthane. Cette réaction est également catalysée par les bactéries mais les mécanismes restent mal compris (Barkay et al. 2003).

Il existe également une photo-dé-méthylation du méthylmercure par la lumière mais elle est faible comparée à la dé-méthylation bactérienne (Parker et al, 2005).

4.3.3 Les bactéries méthylantes

Les bactéries Sulfato-Réductrices ou SRB sont les premières bactéries connues pour être capables de méthyler le mercure (Jensen and Jernelov 1969).

Elles sont présentes dans les sédiments des lacs et des océans et peuvent donc méthyler le mercure divalent présent dans l'eau.

Ce processus se fait uniquement en anaérobiose et à des taux relativement bas.

Notons que toutes les SRB ne sont pas capables de réaliser cette méthylation, les plus aptes sont celles du genre *Desulfobacteriaceae*.

Le processus exact de la méthylation ainsi que son schéma enzymatique est loin d'être compris mais il semblerait qu'elle se fasse à partir du sulfure de mercure HgS contenu dans le sédiment. Le groupement CH_3 serait quand à lui issu de la sérine ou du formate provenant du cycle de l'acétylCoA, la méthylation serait ensuite catalysée par la méthylcobalamine (Barkay et al. 2003).

En aérobiose on n'a jamais observé de méthylation bactérienne.

Notons qu'il existe une méthylation abiotique du mercure. Elle est possible grâce à des acides humiques et fulviques, des acides carboxyliques ou des composés alkylés mais les taux de methylation sont très faibles de l'ordre de 0.01 à 0.1% du mercure divalent présent (Falter, 1999).

4.3.4 Les levures et le mercure

Les mécanismes de résistances envers les métaux chez les levures sont moins bien connus que chez les bactéries.

Néanmoins on a pu observer chez *Saccharomyces cerevisiae* la présence d'une protéine capable de détoxifier le mercure en le concentrant dans la vacuole (Gueldry et al, 2003).

Ce système de résistance est basé sur la reconnaissance de complexes métal-gluthation, une fois le complexe formé il est transporté dans la vacuole ce qui empêche la cellule d'être intoxiquée.

De plus, il semble que de fortes affinités existent entre la paroi des cellules de levures et les métaux (Kungolos et al, 1999).

Finalement, certaines études ont montrées que des levures comme *Saccharomyces cerevisiae* et *Candida albicans* pouvaient produire des espèces organomercurielles lorsqu'elles étaient incubées dans un milieu contenant du mercure. Mais la nature exacte de ces molécules n'a pas été investiguée à l'époque (Yannai et al, 1991) et ce phénomène n'a pas été observé depuis.

5. English Summary of the chapter 2

Mercury was used since antiquity. It has several utilities. For example, under its elemental form because of its amalgams properties, it was used to extract silver and gold.

Under its divalent form it is biocide and antiseptic. Mercury is the unique liquid metal and exists under three oxidation states.

Elemental mercury (Hg^0) is insoluble and volatile. It has a long residence time in the atmosphere (about 1 year) and its atmospheric concentration is homogeneous all around the earth between 1 and 4 ng/m^3 of air (1,7 ng/ m^3 in the north hemisphere).

This elemental form could be oxidised in the divalent form Hg^{2+}. Which is soluble and could be washed by precipitations. This is the form usually found in atmospheric waters.

The divalent mercury could be integrated in the ecosystems where it could be methylated by biotic process.

Mercury had both natural and anthropogenic origins, it results of the volcanic activity or of the erosion of soils. It also have an anthropogenic origin

because of different human activities like combustion of fossil fuels. Anthropogenic emissions represents more than half of the total mercury content in the atmosphere.

Mercury have different toxicity depending on its form. Hg^0 is relatively inoffensive, it could just causes soreness in the respiratory system by a direct and concentrate inhalation. At high levels Hg^{2+} could cause neurological and psychiatric problems. But the most dangerous form of mercury is the organic one: methylmercury which is more lipophile and could bio-concentrate in the food chains. This type of contamination led to dramatic neurological disease for animal or humans at the top of the food chains.

The mercury cycle in the environment is illustrated by Figure 2.2 in Chapter 2. Elemental mercury could react by several pathways to be oxidised in its divalent form. It is subjected to wet and dry deposition. Divalent mercury could be methylated by bacteria in anoxic sediments.

In Arctic, mercury have a special behaviour. Because of a particular chemistry, some mercury is deposited on the snow surface every spring. This phenomenon was called "Atmospheric Mercury Depletion Events". It led to the deposition of mercury species onto the snow. The detailed of the species deposited are presented in chapter 2 (Part 2.2.2).

Arctic ecosystems are known to be contaminated by mercury since several decades. The arctic food chain is presented in the chapter 2 by figure 2.6. Basically phytoplankton is eaten by zooplankton. Zooplankton is consumed by fishes like sculpin, arctic char or arctic cod. Fishes are consumed by seals which could be eaten by polar bears. This food web is contaminated by mercury from plankton to polar bears.

Some recommendations exists on fish consumption for pregnant women, children and elder people, the US Food and Drug Agency poses an exposition limit at 1µg/g of fresh fish.

In any case, in a food web, micro organisms play an important role by recycling organic matters and waste. The resulted nutrients contribute to the primary production of the ecosystem. This pathway is not very important in terms of total carbon mass but could be important in the transfer of pollutant like mercury. That's why we focused on micro organisms.

In the environment, micro organisms are ubiquitous, they could live in several extreme environment like hot sources or in saline media. They could also live in cold environment.

Several studies about microbial content in extreme area exists. They show that micro organisms could be isolated from sea ice, glaciers and snow. The mains bacterial species belongs to Proteobacteria genus, Cytophaga-Flavobacterium-Bacteroides genus and Actinobacteria genus. Some yeast and fungi could also be isolated from snow.

Micro organisms are known to be able to react with different pollutant and for mercury some resistant bacteria exists. In mercury contaminated environment, resistant bacteria are able to reduce divalent mercury to elemental mercury through some resistance genes: the *mer* genes. The details of the resistance mechanism is presented in chapter 2 by Figure 2.5. Divalent mercury is linked to a first protein in the périplasme MerP which present it to another protein MerT.

MerT transmit this mercury in the cytoplasm to a reductase MerA which reduce it to elemental mercury. Then this elemental mercury go back to the atmosphere.

Another reaction which could be mediated by bacteria is the demethylation of mercury. This mechanisms seemed to be link to the resistance genes but is not present in all the resistant bacteria.

Some bacteria could also methylate mercury. They belongs to the Sulfato-reductant Groug and are called Sulfato-Reductant Bacteria (SRB). They are present in the sediments from lakes and ocean and they could methylate mercury under anaerobic conditions. Even if the productivity of this pathway is low, the methylmercury formed could enter the food web via microscopic organisms and be concentrate at every level of the chain. The exact mechanisms of methylation by bacteria is not clearly understood.

Some interaction between yeast and mercury also exists. Some of them seemed to be able to resist to mercury but the exact physiological phenomenon is still unclear.

All this knowledge led us to investigate the role of the microbiological component in the mercury cycle in Arctic snow.

We have first isolated, cultivated and identify 10 arctic bacteria and 3 yeast. Then we tested their behaviour with mercury and methyl mercury.

We also investigate the mercury content in biofilms collected in Arctic and Antarctic and in planktonic samples collected during a field campaign in Ny-Ålesund (Svalbard) in spring 2007.

Chapter 3: Methods

1 Presentation of the different sampling sites

1.1 Snow sampling in Ny-Ålesund

Ny-Ålesund (78°56'N; 11°52'E) is a scientific village located in the North West of Spitzberg which is the largest Island of the Svalbard. This island group is related to Norway.

Ny-Ålesund is our studying site to work on AMDE and our team worked over there for 5 years, by doing field campaign during springs (2004, 2005, 2007 and 2008).

Figure 3.1 presents the location of Svalbard and Ny-Ålesund.

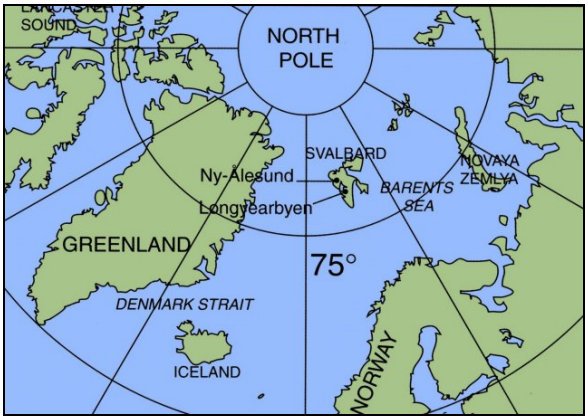

Figure 3.1: Location of Svalbard and Ny-Ålesund

The snow from which the micro organisms were isolated was collected during a previous campaign in 2004. Snow collection was performed in a snow field which was apart from the scientific base close to the shore, next to a cabin where Hg^0 was measured continuously. Figure 3.2 presents the snow field were the snow was sampled.

Figure 3.2 : Picture of the sampling field in Ny-Ålesund.

In spring 2007, I took part to a two months field campaign in Ny-Ålesund. During this campaign gaseous elemental mercury in the atmosphere was monitored, such as inorganic mercury in the snow. The techniques used to do these measurements are presented in the following parts (1.2 and 1.3). During this campaign planktonic samples were also sampled every week, the details of the sampling are presented in part 1.4.

1.2 Total mercury measurements in snow

Once sampled in clean bottles (see part 5.1 for details of washing procedure) snow was melt and then spiked with BrCl to assess 0.5% (w/w) final concentration and let in the dark during one night. This step permits to dissociate all the mercury complexes and to have all mercury species under the divalent form Hg^{2+}.

Total mercury analysis were performed on a Tekran liquid phase analyser (Model 2600, Tekran Inc). This instrument permitted to concentrate mercury on gold traps which were heated to desorbs mercury and detect it as Hg^0 by atomic fluorescence spectrometry (λ= 253,7 nm). The detection limit of this method is better then 0,5 $ng.L^{-1}$

1.3 Mercury measurements in Ny-Ålesund air

To analyse the gaseous elemental mercury in Ny-Ålesund atmosphere during the field campaign a Tekran gas-phase mercury vapor analyzer (Model 2537A, Tekran Inc) was used.

This technique is based on collection of ambient mercury on gold traps followed by thermal desorption and final detection as Hg^0 by atomic fluorescence spectrometry (λ= 253,7 nm).

The detection limit of this instrument was 0,1 ng/m^3 of air.

1.4 Planktonic sampling in Kongsfjorden

The planktonic samples were collected in the fjord of Ny-Ålesund : the Kongsförden, every week between the 25th of April and the 16th of June during the 2007 field campaign.

The Chlorophyll peak was identified with a CTD (Conductivity Temperature Depth) probe to determine the sampling depth.

Then the samples were collected with a 500 µm net and then separated in 5 fractions (i.e. > 500 µm, 500-200µm, 200-63 µm; 63-30 µm;<30 µm).

Once separated the fraction were conserved at –20°C before being freeze dried and analysed.

This work was done with Stéphane Gasparini, Fanny Narcy and Margaux Noyon from the Laboratoire d'Oceanographie de Villefranche (LOV).

1.5 Biofilms and biological samples

After a conference in 2006 in Innsbruck ("International Conference of Alpine and polar Microbiology") a collaboration with two Spanish teams (A. Camacho, University of València and A. Quesada University of Madrid) who worked on biofilms and biological samples in Arctic and Antarctic started.

The aim of the work was to analyse mercury content in such kind of samples and also to test if biofilms exposed to mercury were able to methylate or to store it.

The microbial mats were all non marine samples, and were sampled in small lakes or ponds in Arctic or Antarctic. Assuming that a pond is a body of water smaller than a lake, where light penetrates at the bottom.

They were numbered from 1 to 12, 3 of them were sampled in Arctic and 9 of them in Antarctic. Finally samples of lichens, moss and soil were also taken.

The details of the samples were presented with the results, under the form of a table, in chapter 11 (Table 11.1).

Their exact locations are presented in the following part on figures 3.3, 3.4 and 3.5a and b.

1.5.1 Location of biofilms sampling sites

Five biofilms were sampled in ponds located on an ice shelf close to MacMurdo Scientific Station (i.e. Mac Murdo Ice Shelf which is a 1500 km² area containing variety of small lakes and ponds) in Antarctica (samples 2, 3, 4, 5 and 7). Figure 3.3 presents the localisation of this scientific base, the figure also underline the location of the South Shetland Islands where some other samples are originated.

Figure 3.4

Figure 3.3 : Location of the Mac Murdo Station in Antarctica and of the South Shetland Island (see Figure 3.4 for details).

One sample (sample 1) came from a lake of Hope Bay which is located on Trinity peninsula, another one was sampled in Deception Island (sample 6) which belongs to the South Shetland Islands and two other samples came from Byers peninsula on Livingstone Island (samples 11 and 12) such as lichens, soils and moss samples. These location are presented on Figure 3.4.

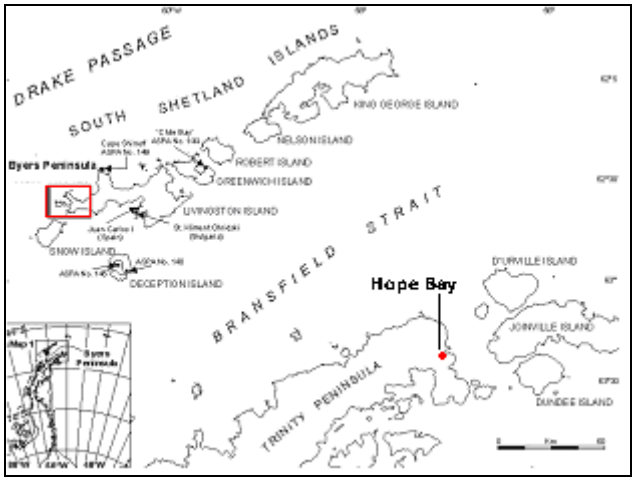

Figure 3.4 : Location of Hope Bay, Deception Island and Byers peninsula in Antarctica

Finally three biofilms (samples 8, 9 and 10) were sampled in the Ward Hunt Ice Shelf, which is the largest ice shelf in the Arctic, located on the north coast of Ellesmere Island, Canada. Figure 3.5a presents the location of this ice shelf and Figure 3.5b presents the lake C1 where samples 9 and 10 were taken.

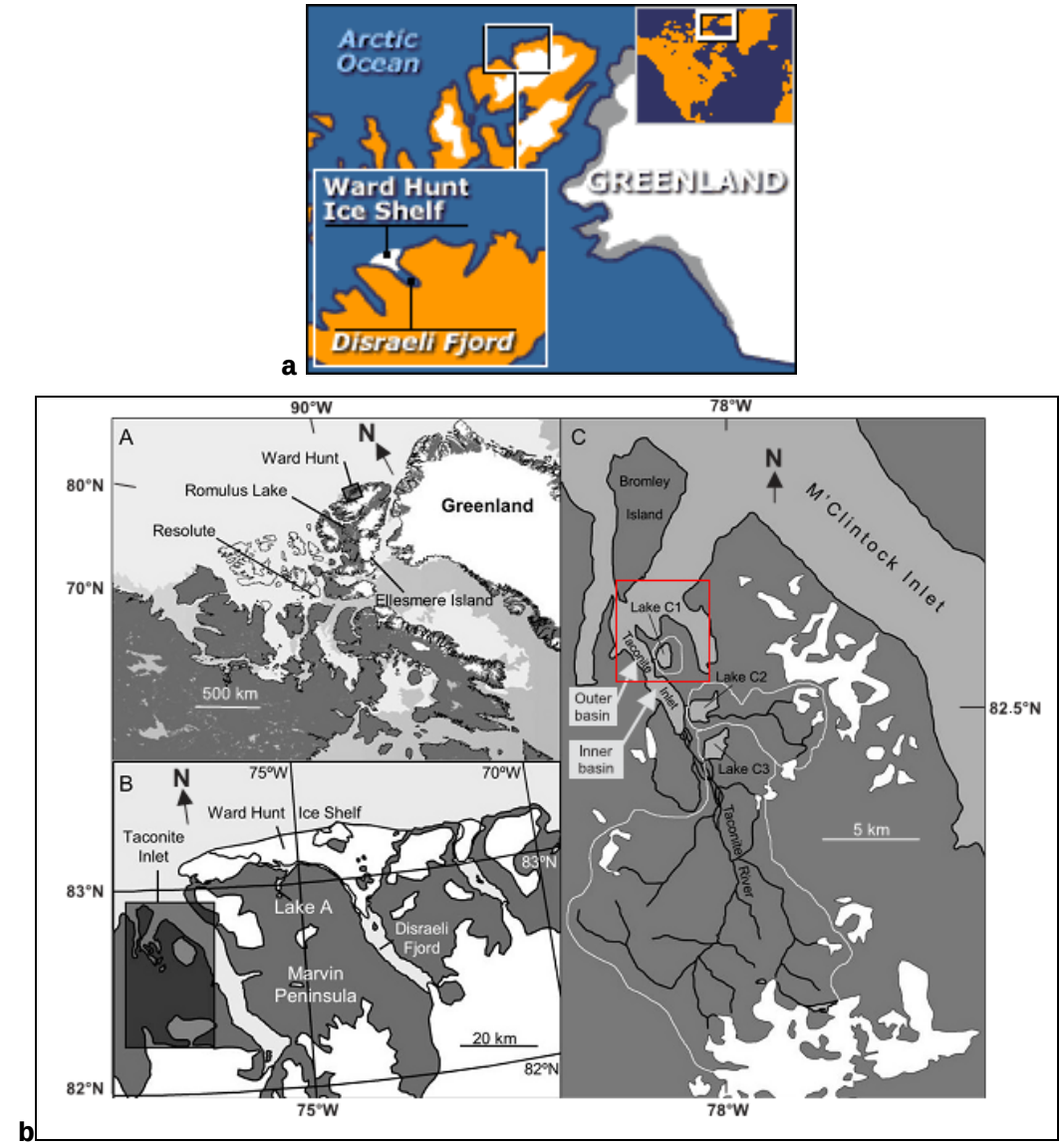

Figure 3.5 a, b : Location of the Lake Ward Hunt in Arctic (a), and location of the lake C1 (b)

1.5.2 Exposition of an Antarctic biofilm to inorganic mercury

In order to determine the potential for Hg accumulation and an eventual methylation by the biofilms, a microbial mat from Suoreste lake (located in the south-western part of Byers Peninsula), was exposed during 50 hours to different concentration of soluble mercury (0, 50, 200, 500, 1000, 5000 ng/L of $HgCl_2$).

Pieces of the biofilm were cut and fitted to the bottom of 50 ml acid-washed glass vials, filled up with the water covering the mats, and supplied with adequate amounts of the stock mercury solution. Each vial was gently shaken every four hours during the incubation, which lasted for 50 hours.

After incubation with mercury microbial biofilm were washed three times with ultrapure water and stored frozen until total mercury and methyl mercury analysis presented in part 6.3 of this chapter.

2 Microbiological methods used during the PhD

The part 2.1 and 2.2 are extracted from the publication "Bacterial characterization of the snow cover at Spitzberg, Svalbard" written by Amato, et al, 2007, this paper is included in the chapter 4, but for more visibility the methods used are also described here.

All microbiological manipulations were performed in the Laboratoire de Synthèse Et Etude des Systèmes à Intérêt Biologiques of Clermont Ferrand, France (SEESIB, Université Blaise Pascal).

2.1 Isolation of the micro-organisms from snow

To begin, the first layer of snow that was in contact with air was removed with a sterile spoon. Then the snow was collected with a sterile tube and was conserved frozen until arrival in the microbiology lab.

It was then slowly melted under sterile conditions and plated on solid TS and solid R2A. The snowmelt was also mixed with liquid TS and Liquid R2.

Once growth was detected 0,1 mL was plated on the corresponding solid medium. The differentiation of the strains was done on morphological criteria.

Each sample to be used for cultivation was stored and transported frozen from Ny-Ålesund to the laboratory in France for analysis. It was then slowly and completely melted at ambient temperature, and then divided to be incubated under different conditions.

First, triplicates of 0,1 mL were directly plated onto solid R2A and trypcase soy (TS) media supplemented with agar (20 $g.L^{-1}$), to be incubated at 4, 15 and 27 °C.

By contrast, liquid cultures were performed by enrichment of the melted snow with TS (1% and 50% v/v final concentration of the nutritive medium initially prepared at 30 $g.L^{-1}$) and R2 (1% and 50% v/v final concentration of the nutritive medium initially prepared at 3.2 $g.L^{-1}$) media.

Liquid R2 was prepared according to the recipe of the commercial medium R2A presented in the following table. The TS medium came from BioMérieux, France, its composition il also presented in table 3.6.

R2 Medium	
Yeast extract	0,5 g/L
Proteose peptone	0,5 g/L
Casamino acids	0,5 g/L
Glucose	0,5 g/L
Soluble Starch	0,5 g/L
Potassium pyruvate	0,3 g/L
Phosphate dipotassium	0,3 g/L

TS Medium	
Tryptone	17 g/L
Soytone	3 g/L
NaCl	5 g/L
K_2HPO_4	2,5 g/L
Glucose	2,5 g/L

Figure 3.6: Composition of the R2 and the TS media

Triplicates of 100 mL of all these four media were made in 500 mL flasks incubated at 17 °C, under agitation (200 r.p.m.).

When growth was visually detected, 0.1mL was plated onto the corresponding solid medium for isolation of colonies.

Each colony was differentiated from the others on the basis of morphological criteria, and isolations were ensured by successive transfers onto the same medium if necessary.

For all the experiments, prechilled filter tips and media were used to preserve cells from any heat shock.

This part is also illustrated in the figure 3.7.

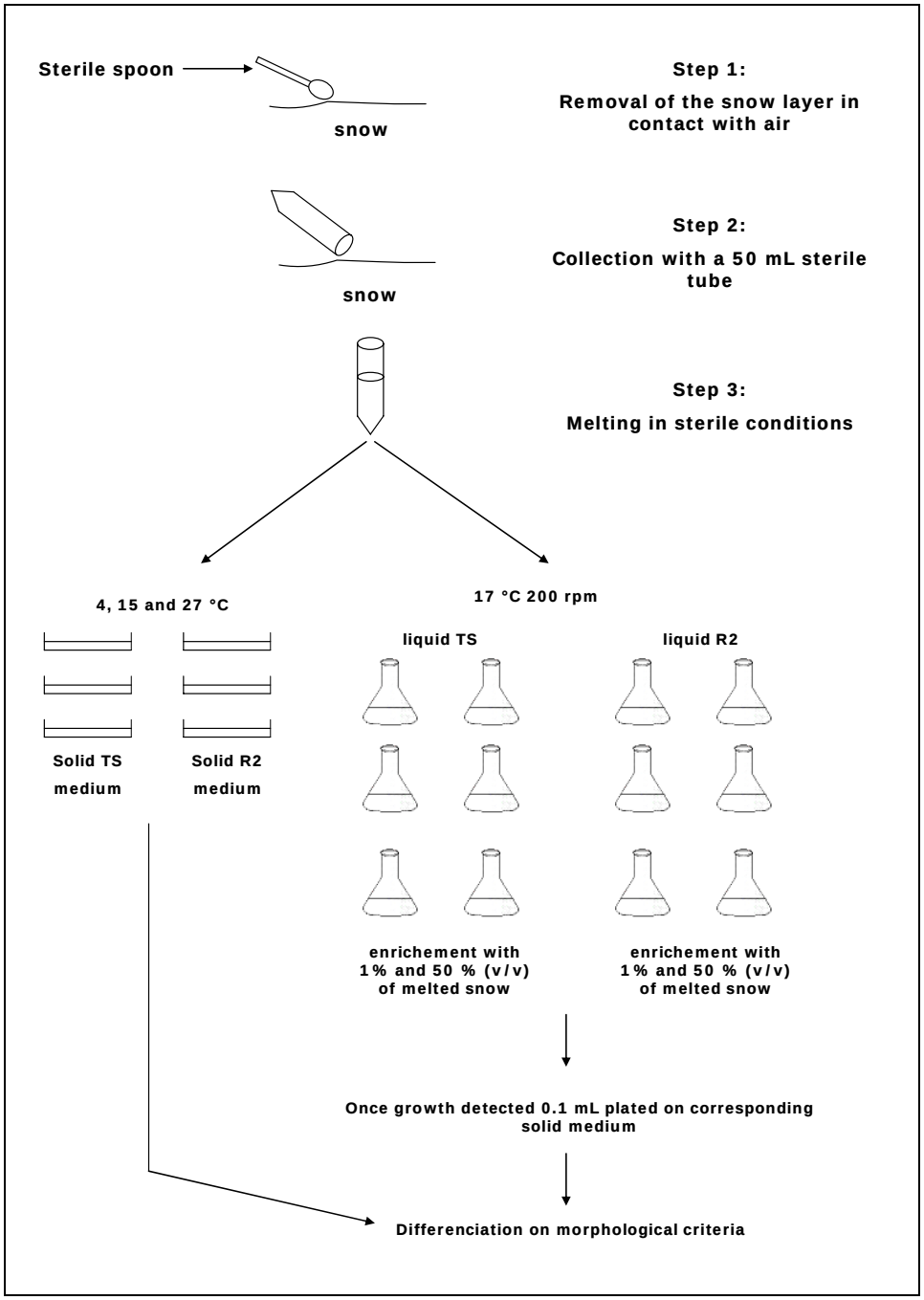

Figure 3.7: Representation of the procedure used to isolate micro organisms from snow

2.2 Identification of the microorganisms

2.2.1 Bacteria

This identification was performed by sequencing 16S RNA. RNA extaction was performed as follow. Cell pellets obtained after centrifugation of pure liquid cultures of the isolated strains were re-suspended into phosphate-buffered saline (PBS) solution and their total genomic DNA was extracted using the Easy DNA Kit (Invitrogen).

Extracts were checked by gel electrophoresis, and 16S rRNA genes were amplified by PCR. This step was carried out using universal primers for Eubacteria: F8-Eub (5'-AGA GTTTGATCMTGGCTC-3') and 1492r-Univ (5'-GNTACCTTGTTACGACTT-3') (Humayoun et al. 2003), in which M corresponds to A or C, and N to any one of the four nucleotides.

About 100 ng of genomic DNA and 1.5 U of Taq polymerase (QBiogene) were employed.

PCR was performed as follows: 25 cycles of 30 seconds at 94 °C for DNA, 30 seconds at 55 °C and 90 seconds at 72 °C, preceded by 5 min at 94 °C and ending with 7min at 72 °C. PCR products were checked by gel electrophoresis, and purified using a Strataprep purification kit (Stratagene).

They were finally freeze dried and sequenced by capillary electrophoresis (MWG-Biotech), using the previously described F8-Eub and 1492r-Univprimers, and a central primer, F500 (5'-CTAACTACGTGC-CAGCAGC-3'), in order to obtain overlapping sequences.

Comparisons of almost complete reconstructed 16S rRNA gene sequences with those included in GenBank were performed using the BLASTN interface, available at http://www.ncbi.nlm.nih.gov/BLAST/. Multiple alignments of the 16S rRNA gene sequences obtained with some selected from among the closest related neighbours and others selected in the literature were achieved using CLUSTALW (http://www.ebi.ac.uk/clustalw/) and visually checked.

Lengths of sequences were adjusted according to these multiple alignments and final lengths of 1367 and 1386 bp were thus compared for Gram-negative and Gram-positive isolates, respectively. One hundred trees generated by bootstrap were analysed by the neighbour joining method of the PHYLIP 3.63 package (Felsenstein 2004)after application of the Kimura two-parameter correction on calculated distances (correction of differences between

rates of tranversion and transition) (Kimura 1980). Two consensus trees were finally constructed to place both our Gram-negative and Gram-positive isolates.

2.2.2 Yeast

Yeast were identified by CBS (CentraalBureau voor Schimmelcultures i.e. Fungal Biodiversity Centre, Netherland, http://www.cbs.knaw.nl). The identification was based on morphological criteria and comparison to known species.

2.3 Growth of micro-organisms

The growth of each strain was followed by measuring the Optical Density at 575 nm.

Each strain was cultivated at 5, 12, 17 and 27°C in R2 liquid media under 200 rpm agitation in triplicates and possible contamination by other micro organism was checked by plating on solid R2 medium.

Growth curves (i. e. OD_{575} = f(t)) permits to determine the optimal growth temperature.

3 Minimum Inhibition Concentration measurements (M.I.C.)

Minimum Inhibition Concentration corresponds to the minimum concentration of a pollutant which inhinits micro organisms growth.

M.I.C. measurements were performed in triplicates by testing mercury as mercury chloride ($HgCl_2$) in R2 liquid medium at 17°C under 200 rpm agitation, during 72 hours for both bacteria and yeasts.

MIC measurements on bacteria were performed by N. Maruczak during his master 2 (Marusczak 2007) and MIC measurements on yeast were performed by J. Rosset during his master 1 training period that I supervised.

4 Investigation of the resistance of bacteria toward mercury

The resistance phenomenon in bacteria was also investigate on the 10 bacterial strains. It is based on synthesis of different protein which will reduce divalent mercury to its elemental volatile form.

All the proteins included in this mechanism are encoded by the *mer* operon presented with more details in chapter 2.

To investigate the presence or absence of *mer* genes in Arctic bacteria, a portion of the resistance genes was amplified with Polymerisation Chain Reaction (PCR).

This work was started during my master 2 and at the end performed in the Laboratoire d'Adaptation et de Pathogénie des Microorganismes de Grenoble by N. Maruczak during his Master 2 (Marusczak, 2007).

4.1 Primers used for amplification and PCR conditions.

4.1.1 For Gram negative bacteria

4.1.1.1 Detection of *merA* genes

For gram-negative Arctic strains (*Chelatoccocus sp., Brevundimonas sp. Hydrogenophaga sp., Moraxella sp., Sandarakinorhabus limnophila 94.5 and 95.2%*), bacterial genomic DNA was extracted and quantified before PCR.

Primers used where universal ones for Gram negative, already described by(Chadhain et al. 2006), their sequences were :

- wA1s-n.F: 5'-TCCGCAAGTNGCVACBGTNGG-3'
- wA5-n.R: 5'-ACCATCGTCAGRTARGGRAAVA-3'

Where V corresponds to G, A or C; B corresponds to G ,T or C, R corresponds to G or A and N correspond to one of the four nucleotide.

These primers permit to amplify a *~285 bp* DNA fragment of the merA gene.

PCR was performed as follows: 45 cycles of 10 seconds at 94 °C for DNA, 60 seconds at 54 °C and 60 seconds at 72 °C, preceded by 3 min at 94 °C and ending with 10 min at 72 °C.

4.1.1.2 Detection of *merR* genes

For *merR* genes of Gram negative bacteria primers for very conserved area of the gene *merR*.

- wmerRTP1: 5'-GGGAGATCTAAAGCACGCTAAGGC(A/G)TA-3'
- wmerRTP2: 5'-GGGGAATTCTTGAC(G/A/T)GTGATCGGGCA-3'

The PCR was performed as follows 30 cycles of 30 seconds at 94 °C for DNA, 60 seconds at 55 °C and 120 seconds at 72 °C, preceded by 3 min at 94 °C and ending with 10 min at 72 °C.

These primers permit to amplify a *~1016 bp* DNA fragment of the *merR* gene.

To control the gene amplification, genomic DNA from the resistant Gram positive bacteria *Cupriavidus metallidurans CH34*, was used as a positive control. This positive control permitted to check the efficiency of the primers and to adjust the PCR conditions.

PCR products obtained were analysed by electrophoresis on 0.8 or 2% agarose gel. DNA was revealed by an Ethidium Bromide coloration which fluoresces with ultraviolet (280-320 nm).

4.1.2 For Gram positive bacteria

For gram-positive Arctic strains, (*Bacillus sp.*, *Micromonospora sp.*, *Paenibacillus sp.*, *Agromyces sp.*) PCR were performed directly on a cellular suspension and the detection was performed for *merA* genes

Primers used where those described by Hart et al, 1998. They amplify a ~ 1300 bp fragment of *merA* gene.

Their sequences were:

- wMerAbac1: 5'-TGGGTGGAACTTGCGTTAA-3'
- wMerAbac2: 5'-GTA(GT)CC(TA)GCACA(GA)CAAGATA-3'

The PCR was performed as follows 30 cycles of 60 seconds at 94 °C for DNA, 60 seconds at 53 °C and 120 seconds at 72 °C, preceded by 3 min at 94 °C and ending with 10 min at 72 °C.

All PCR products obtained were analysed by electrophoresis on 0.8 or 2% agarose gel. DNA was revealed by an Ethidium Bromide coloration which fluoresces with ultraviolet (280-320 nm).

4.2 Cloning of PCR products obtained

To control and conserved the PCR products obtained, they were cloned in a plasmid before being sequencing.

Plasmid used was pCR®II-TOPO® (Invitrogen™) and is presented in figure 3.3.

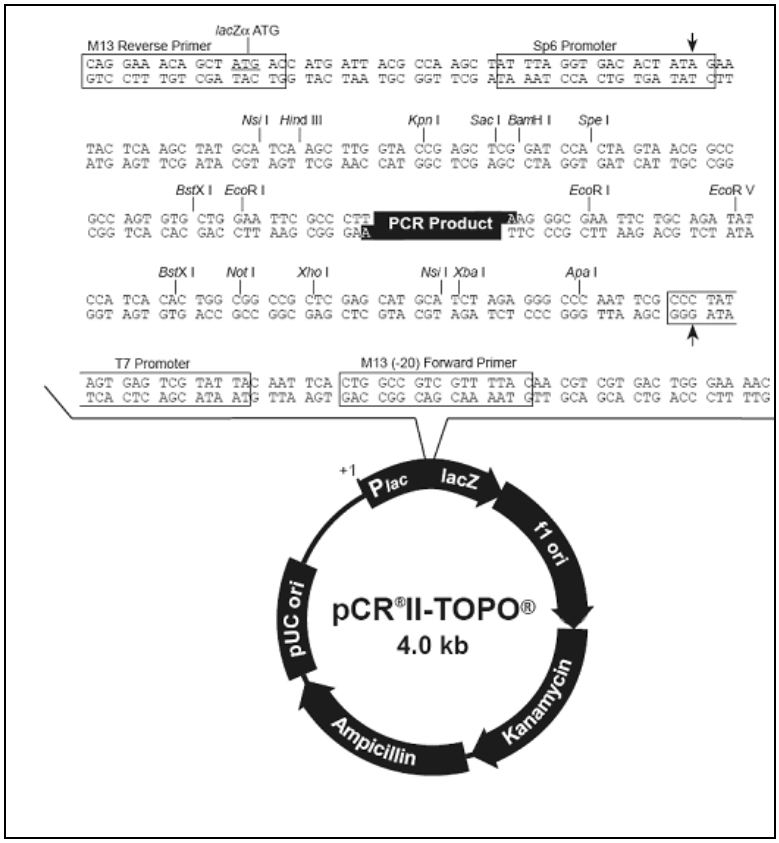

Figure 3.8: restriction map of plasmid pCR®II-TOPO®

Cloning was done following the manufacturer's instructions.

Ligation mixtures were transformed into competent *E. coli* TOP 10 (Invitrogen™). Clones carrying recombinant plasmid were selected on LB-kanamycine (40 µg.mL^{-1})- Xgal (40 µg.mL^{-1}).

Kanamycine permited to select colonies containing a plasmid, and X-gal permitted to discern plasmid with insert colonies from plasmid without insert ones.

As illustrated by figure 3.9, PCR products were inserted in the *lacZ* gene, this gene coded for an enzyme: β-galactosidase.

If the plasmid contained the insert of the PCR product (= recombinant plasmid), the *lacZ* gene will not be functional. So the colonies carrying the recombinant plasmid will not express β-galactosidase anymore and will be white

on this kind of medium. The colonies carrying a non recombinant plasmid had an active β-galactosidase and were blue.

Once selected white clones were cultivated in 2 mL of LB-kanamycine medium at 37 °C during one night. Then the plasmid pCR®II-TOPO® was extracted with a extraction kit (GeneJET™ Plasmid Miniprep Kit, Fermentas) before being sequenced with primers Sp6 et T7 specific of the plasmid pCR®II-TOPO®.

5 Interaction between micro-organisms and mercury

5.1 Washing procedure for trace metal work

To continue the work about micro organisms and mercury an interaction experiment was designed.

As the levels found in normal water could be a thousand times higher than the ones observed in Arctic.

Working with such levels (ng/L) of mercury required extra attention to glass cleaning to avoid contamination by extra mercury.

Glass cleaning procedure was performed in a clean room (class 10 000) and followed a strict protocol already described by Pierre-Alexis Gauchard (PhD thesis) and presented in the Table 3.10.

Step 1	Rinse with soapy water
Rinse with ultrapure water 3 times	
Step 2	4 days in 10 % ultrapur Nitric Acid
Rinse with ultrapure water 3 times	
Step 3	4 days in 1% ultrapur Hydrochloric acid
Rinse with ultrapure water 3 times	
Step 4	4 days with BrCl 0.5 %
Step 5	Neutralization of BrCl with NH_2OH
Conservation filled with ultrapure water	

Table 3.10 : Washing procedure concerning the glass work.

The acids used were Nitric acid (HNO_3 69% RP Normapure for mercury traces analysis, VWR) and Hydrochloric acid (HCl 30%, Normatom ultra-pure, VWR).

BrCl was prepared in a teflon flask as follows. The first step was to dissolve 1,1 g de $KBrO_3$ (RP Normapure, VWR) and 1,5g of KBr (RP Normapure, VWR) in 20 mL of ultra-pure water. The second step was to add slowly 80 mL of Hydrochloric acid (HCl 30%, Normatom ultra-pure, VWR). This step was done under an extraction hood because of the formation of Cl_2 and Br_2 vapour.

The NH_2OH solution was prepared by dissolving 30 g of NH_2OH (for trace metal analysis, Merck) in 70 g of ultrapure water.

The levels of mercury in the ultrapure water were under the detection limit of the instrumentation.

5.2 Experiment to investigate the behaviour of micro-organisms with environmental levels of mercury

5.2.1 Presentation of the experiment

After the first results obtained during my master 2 which indicated that growth of bacteria and yeasts were not disturbed by environmental amount of mercury (100 ng/L) and that in a pure culture of bacteria inorganic mercury disappeared totally from the media within few days, another experiment to study more carefully the phenomenon observed was elaborated.

Indeed, the experiment performed during the master consisted in a spike of mercury in a pure culture of bacteria or yeast, sampling at different times and a removal of bacteria from the growth medium by centrifugation. The first idea was to analyse mercury content in the cell pellets after an extraction with 10% hydrochloric acid and 10 min sonication but the pellets obtained were too small to permit an analysis.

So another way to separate micro organisms from growth medium needed to be found.

We adapted the protocol of Mirimanoff et al. 2000 who worked on interaction between bacteria and zinc.

The idea was to cultivate each micro organims in pure culture containing amount of mercury close to those deposited by AMDE and to follow the fate of mercury in the medium, on the cell wall and inside the cells.

To manage to analyse these three fractions the experiment was performed as follows.

A 500 mL flask filled with 200 mL of R2 liquid medium was inoculated at 1% with a preculture (OD_{595}=1) of bacteria or yeast. Then this pure culture was spiked with ^{202}mercury (100 ng/L).

The experiment lasted 4 days and an aliquot was sampled at different times :

- before mercury addition (control)
- just after mercury addition (0 hour)
- 3 hours after addition
- 24 hours after addition
- 48 hours after addition
- 96 hours after addition

The sample was filtrated on cellulose acetate membrane filter 0,22µm (Fisher Bioblock) to remove bacterial cells. The liquid obtained was called fraction 1.

Then the filter containing bacteria or yeast cells was rinsed by a solution of EDTA 20 mM (Diiammonium Ethylene Diamine TetrAcetate, Sigma Aldrich, France) with a time contact of at least 30 seconds to remove mercury from the cell wall of the microorganisms.

As introduced before, the protocol from Mirimanoff et al. 2000 which inspirited the experiment was created for bacteria and zinc, the stability constant of the complexes EDTA-Zn and EDTA-Hg are close respectively 16,5(Vohra et al. 1963) and 15,3 (Nambiar et al. 1996), indicating EDTA has a high affinity for Hg such as for Zn. Moreover Fein et al. 2001 estimate the stability of complex formed by Hg^{2+}-carboxyl of the cell wall for *Bacillus subtilis* was 9,7, this indicates that EDTA remove mercury bound to the cell wall.

This EDTA rinse correspond to the fraction 2.

At the end the filter was digested by 10% hydrochloric acid (Merck, Italy) and 10 min sonication to obtain the fraction 3.

Figure 3.11 presents a scheme of the experiment performed.

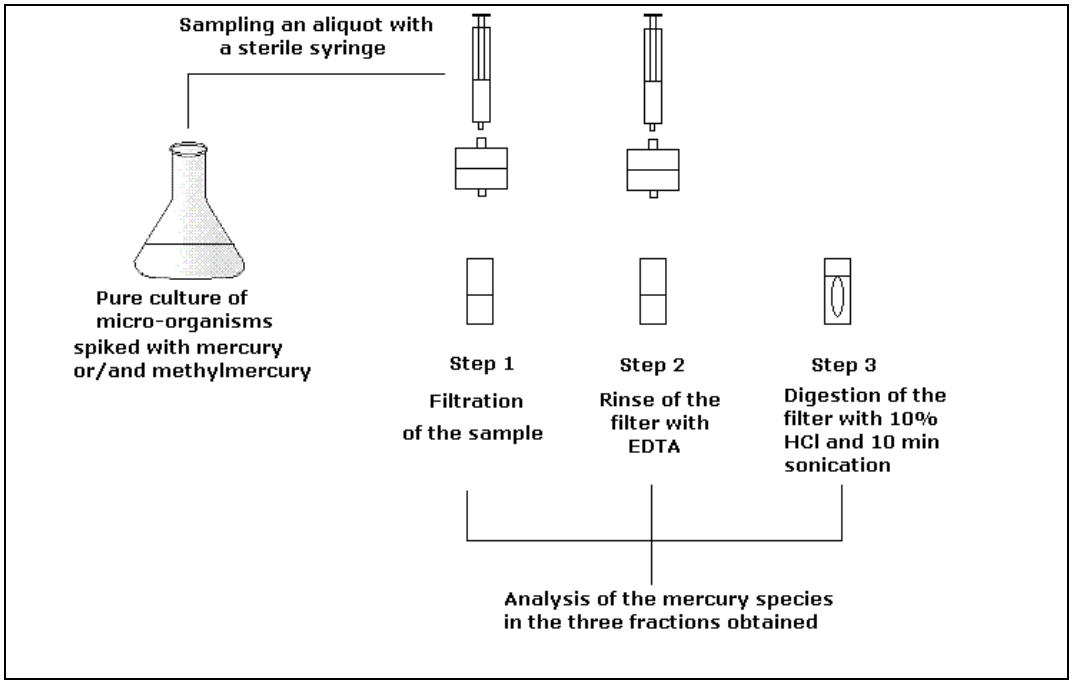

Figure 3.11: Scheme of the interaction experiment between micro-organisms and mercury species.

After this experiment, mercury speciation was measured by HPLC coupled to ICP-MS in each obtained fraction.

This experiment permitted to investigate the behaviour of mercury:

- fraction 1 gave the mercury content in the medium
- fraction 2 gave the mercury content on the cell wall of the micro-organisms
- Fraction 3 gave the mercury content inside the cells

Special care was taken to avoid mercury contamination, all the glass was cleaned following the mercury free procedure presented in part 2.1.

Pipet tips, syringe, and filter-holder used were in plastic, and on this plastic matter mercury could stay stuck during all the steps before analysis. To solve this problem the system syringe, filter holder and filter were rinsed by 10 mL of 10% diluted HCl and 10 mL of ultrapure water before each sampling.

Between each sample, the filter holder was washed in a bath of BrCl 0,5% and let in the dark.

As pipet tips were first thought to be mercury contaminant, they were rinsed with 10% HCl before sterilisation. It was found later that they bring no extra mercury.

5.2.2 Improvements of the experiment

This experiment was performed several times during the PhD, the next table summarises the different problems which have occurred.

As introduced before, the interaction experiment was performed in Clermont Ferrand and the mercury analysis were done in Venice.

The Table 3.12 summarises the different experiments performed during the PhD, with the results obtained and the solution applied.

<table>
<tr><th colspan="2">Period of experimentation</th><th rowspan="2">Experiment</th><th rowspan="2">Results and problems</th><th rowspan="2">Solution</th></tr>
<tr><th>In Clermont Ferrand</th><th>In Venice</th></tr>
<tr><td rowspan="2">July 2006 and September 2006</td><td>October 2006</td><td rowspan="2">Micro organisms grown with 100 ng/L of ^{202}mercury (10 bacteria and 3 yeasts tested)</td><td>• Defrost of the sample during storage
• Mercury detected since T-1
• Detection of methylmercury for certain samples</td><td rowspan="2">o Focus on yeasts
o Use of isotopes to avoid contamination
o Test of interaction with methylmercury
o Glycerol stock solution thought to be contaminant, plating of the strains on R2 medium and use of an colony to inoculate the culture.</td></tr>
<tr><td>March 2007 (End of analysis of the samples)</td><td>• Confirmation of methylmercury detection only for the yeasts cells</td></tr>
<tr><td>September 2007</td><td>October 2007</td><td>3 yeasts grown with 100 ng/L ^{199}mercury and 150 ng/L methyl^{201}mercury</td><td>• Problem with the methylation protocol applied
• Methylation did not work
• Less isotope amount introduced than expected</td><td>o Optimization of methylation process and exact titration of isotopes solutions</td></tr>
<tr><td>November 2007</td><td>December 2007</td><td>3 yeasts grown with 100 ng/L ^{199} mercury and 150 ng/L methyl^{201}mercury</td><td colspan="2">• Results presented in chapter 8 and 9</td></tr>
<tr><td>February 2008</td><td>March 2008</td><td>4 bacteria grown with 100 ng/L ^{199}mercury and 150 ng/L methyl^{201}mercury</td><td colspan="2">• Problem with isotopes solutions
• Amount of isotopes added higher than expected
• Data not exploitable</td></tr>
</table>

Figure 3.12: Summary of the interaction experiment performed during the PhD

To start we were faced to a freezer problem, which led to the defrost of the samples of July 2006. Because defrost of the samples could allowed a bacterial growth and a mercury speciation change, the results obtained could not be exploited.

Then, on the samples of September 2006 some mercury contamination problems occurred, mercury was detected since the T-1 sample indicated a contamination before mercury addition.

But in these samples methylmercury traces were measured in the samples concerning yeasts which was a very interesting result despite the contamination.

Finally the glycerol stock solution in which the bacteria were conserved and which served to inoculate pre-culture was thought to contain ^{202}mercury.

So to avoid this, for all the other experiments, the strains from the glycerol stock solution were plated on R2 solid medium and one colony was used to start the preculture in R2 liquid medium free of mercury.

This first experiments pointing out the difficulty to work with trace level metal.

To avoid these problems isotopically labelled mercury (201 and 199) was used since September 2007.

Moreover to follow the fate of mercury species during this experiment, it was chosen to spike the culture with inorganic ^{199}mercury to see if a methylation of mercury was possible. And also to spike the culture with methyl^{201}mercury to see if a demethylation phenomenon could occur.

This methyl^{201}mercury needed to be synthetized, this part is presented in the following point.

5.2.3 Methylation of mercury

To methylate ^{201}mercury and ^{199}mercury the methylation was first tested on a solution of ^{202}mercury by following the protocol of H. Hintelman published in 1999.

A 100 µL volume of a 1000 mg/L $HgCl_2$ solution acidified at 10% with HCl was added to 500 µL of Methylcobalamine (Sigma Aldrich, France, 1mg/mL dissolved in sodium acetate buffer 0.1M pH 5)

The mix was kept in the dark during 3 hours at room temperature. The formed CH_3Hg was extracted 3 times with 400 µL of toluene. The extracts were dried over sodium sulfate

But mercury methylation did not occur with this protocol.

After a look in the literature it was thought that pH of the ^{202}mercury solution was too low and led to a problem of disponibility of mercury for the enzyme, so the reaction was tested in water. Moreover, as the methylcobalamine optimum temperature was higher than room temperature the experiment was performed at 30°C.

The influence of the time of reaction and of the volume of enzyme was also tested.

Finally the methylation was tested in two other buffers: sodium acetate 0,01 M and ammonium phosphate 0,1M which permits to assess a final reaction pH of 6.

The best results were obtained with this last buffer at 30°C, with a final concentration of enzyme of 1 mg/mL and a reaction time of 1 hour.

The methylation efficiency was controlled with the HPLC-ICP-QMS.

The Table 3.13 summurises up the different parameters tested to optimize mercury methylation and the percentage of methylmercury obtained.

Final Concentration of MeCob during the reaction (mg/mL)	Solution used for the reaction	Temperature	Reaction Time	% of mercury methylated	Parameter changed
0,5	H_2O	30°C	1h	15	**Temperature**
			one night	25	**Reaction time**
1	H_2O	30°C	1h	37	**Concentration of enzyme**
			one night	69	**Reaction time**
1	Sodium Acetate 0,01M	30°C	1h	46	**Buffer**
1	Ammonium Phosphate 0,1M	30°C	1h	93	**Buffer**

Table 3.13: Summary of the step of optimization of the methylation process

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6 Measurements of mercury species by different techniques

6.1 HPLC-ICP-QMS

This measurement method was used to measure mercury species during the interaction experiment presented in section 3 of this chapter.

All the HPLC–ICP-QMS measurements were performed in the Laboratory of Environmental Sciences of the University Ca'Foscari of Venice (Italy).

As we worked with low mercury levels an accurate and sensitive measurement method needed.

To detect environmental level of mercury to the range of ng/L, ICP-QMS (Inductively Coupled Plasma-Quadrupole Mass Spectrometry) was a good method because of its low detection limit and because it has a signal response independent of the chemical form of mercury (Cairns et al, 2008).

This instrument permits an easy sample introduction as the ion source is at atmospheric pressure and has the accuracy and detection limits of a Mass Spectrometer.

ICP-QMS can perform multi-elemental analysis with excellent sensitivity and high sample throughput.

It is a perfect instrument for analysis of environmental levels mercury as the signal response is independent of the chemical form of a trace element, unlike cold vapour generation where each mercury species has a different instrumental behaviour.

The ICP-QMS instrument employed an argon plasma (ICP) as the ionization source, with an interface to reduce the pressure and ion lenses to transmit the ions to the quadrupole (Q) mass spectrometer (MS) analyzer to detect the ions produced.

It can measure analyte concentrations down to the sub nanogram-per-liter (ng/l) level due to it's high signal to noise ratio and high efficiency ion source.

- **Introduction of the sample**

In an ICP-QMS instrument, the plasma plays the role of the ionization source.

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Liquid samples are typically introduced by a peristaltic pump, to the nebulizer where an aerosol is formed.

A double-pass spray chamber selects the aerosol with an aerodynamic diameter or less than 5 microns and is cooled to 2 degrees to reduce the vapour phase introduced into the plasma. The aerosol is transported to the plasma using an argon carrier gas.

Once in the plasma, the sample aerosol was decomposed to form analyte atoms that are simultaneously ionized.

The analyte ions from the plasma were focused by a series of ion lenses into the quadrupole mass analyzer.

- **Transmission to the mass analyzer and detection**

The quadrupole is constitued by four parallel bars of molybdenum on which a combination of radio frequency and direct current was applied. It played a transmission role between the plasma and the mass analyzer.

The combination of these voltages allows the analyzer to transmit only ions of a specific mass/charge ratio.

Finally, the analyser focussed each ion characterized by a mass/charge ration on the collector. This gave a signal in Counts per Second.

Figure 3.14 presents the different components which constitute an ICP-QMS.

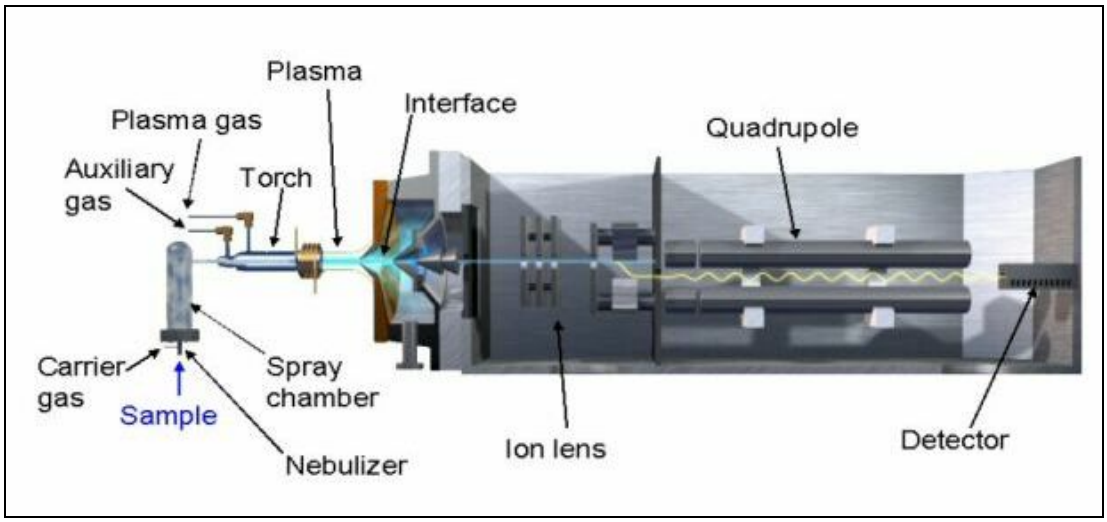

Figure 3.14: Agilent ICP-QMS Block Diagram

The ICP-QMS used during my PhD was an Agilent 7500is (Agilent Technologies, Yokogawa Analytical Systems, Tokyo, Japan) fitted with a standard quartz spray chamber and a PolyPro-ST concentric nebulizer (Elemental Scientific Inc. Omaha, USA). This instrument is presented by Figure 3.15.

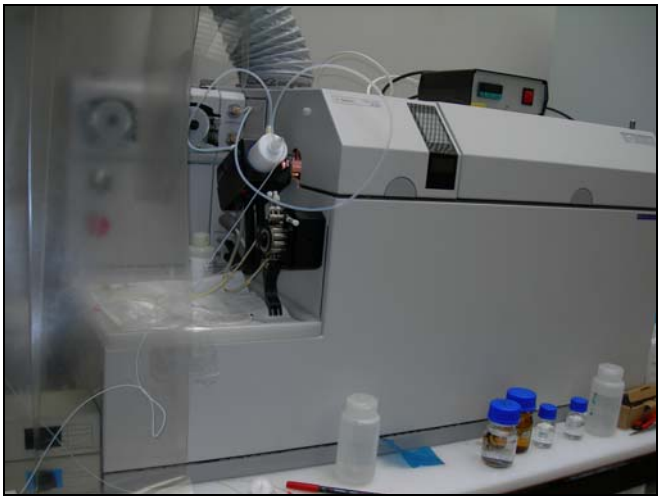

Figure 3.15: ICP-MS Agilent 7500is

6.2 Presentation of HPLC technique

6.2.1 Introduction

To assess mercury speciation a High Performance Liquid Chromatography (HPLC) system was coupled on the ICP-MS.

The use of this system allowed speciation analysis of mercury species without the use of derivatising agent that could cause changes in the species present.

This system allowed to separate mercury species depending of their organic characteristics.

Inorganic species had a shorter Retention Time than the organic forms of mercury (like Methylmercury).

6.2.2 Column used

The column used was a 150 x 4.0 mm HPLC column packed with 5 µm Hypersil ODS C-18 stationary phase (Agilent, Waldbronn, Germany) at a flow rate of 1 mL min^{-1}, with a mobile phase of 0.5 % L-cysteine (m/v) and 0.05 % 2-mercaptoethanol (v/v) dissolved in ultra-pure water.

The sample was introduced with a 0,5mL syringe with a manual injection valve (9125, Rheodyne, CA, USA) fitted with a 200 µL (PEEK) sample loop (Alltech, Deerfield IL, USA) for sample which did not need preconcentration.

The figure 3.16 presents pictures of the column and the sample loop used for these measurements.

Figure 3.16 : On the left : HPLC column and 0,5mL injection syringe. On the right : 200 µL sample loop.

For less concentrated sample an Opti-lynx™ 100 µL microcolumn filled with a C-18 silica based packing material (Alltech, Deerfield IL, USA) was used instead of the 200 µL sample loop to preconcentrate the mercury species prior to analysis. The volume injected onto the preconcentration column was 1mL.

This method separated mercury species as Hg-cysteine ion pairs, meaning that complexes such as complexes of mercury with glutathione and other amino acids with thiol groups are separated and detectable.

This was tested by adding mercury to a solution of glutathione and injecting the resulting solution. A peak for Hg^{2+} and another with a longer retention time was observed demonstrating that Hg-glutathione complexes are detectable with this method.

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Figures 3.17 and 3.18 present a typical chromatogram obtained with this system and the retention times for different mercury species.

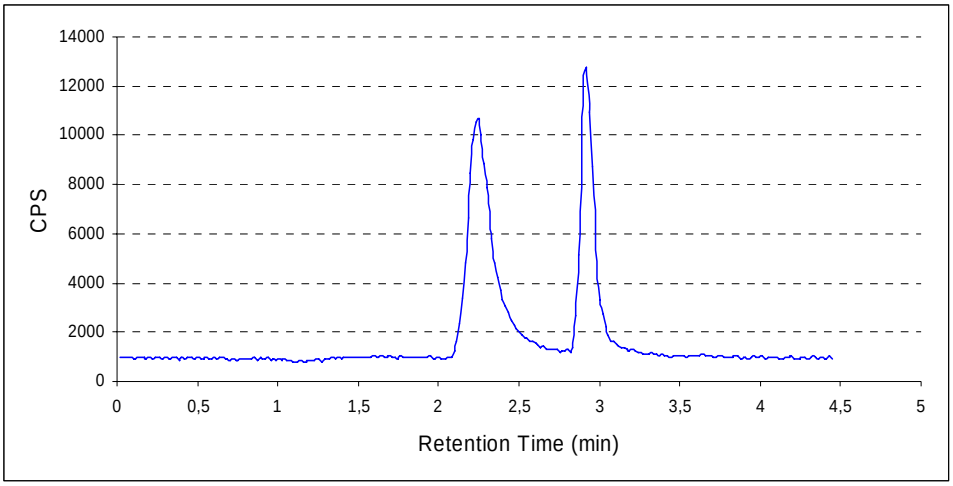

Figure 3.17: Typical chromatogram obtained for inorganic mercury and methylmercury on an HPLC system coupld to ICP-QMS. First peak represented inorganic mercury, second peak represented methylmercury.

Species	Retention Time on the system (min)
Hg^{2+}	2,08
MeHg	2,8
EtHg	6,78
Glutathion Hg	5,61

Figure 3.18: Retention Times of different species on the HPLC column used.

6.2.3 Calibration of the system

We performed a calibration with mercury and methylmercury standards.

The standards tested were 50,100,150 and 200 (ng/L as mercury) and were prepared daily by dilution of a 1 mg/L $Me^{202}Hg$ and ^{202}Hg standard in acidified (10% HCl) ultrapure water.

Standard were measured in triplicate to obtain a Relative Standard Deviation that was always inferior to 10%.

The calibration curves had a R^2 of at least 0,98.

6.3 Measure of Total and Methyl mercury in biofilms, lichen and mosses

These measurements were performed at the GEOTOP of the Université du Québec à Montréal (UQAM) and concerned native biofilms, lichen and mosses sampled in Antarctic and Arctic and an exposed biofilm.

6.3.1 Total Mercury measurements

This method was previously descrbed by Pichet et al, 1999.

Briefly 30 mg of the dried sample was digested with a mix of HNO_3 (16N): HCl (6N) (1mL: 0,1 mL) and heated at 120 °C for 4 hours. After this digestion step the volume of the sample was brought to 3 mL with NANOpure® water, then 0,5 mL were analyzed.

The cold vapour of mercury emitted after a Sn(II) reduction were measured by atomic fluorescence.

A series of digestion included two blanks, one certified standard and then 17 samples including two duplicates. The instrument was calibrated by injecting known amounts of Hg(II) . The detection limit was 0,3 ng Hg/g for 20 mg of dry sample.

The accuracy of the method was estimated by the analysis of the certified standard Mess-3 (marine sediment from National Research Council of Canada) The certified value of Mess-3 was 91± 9 ng/g.

6.3.2 Methylmercury measurements

Methylmercury needed to be extracted from the dried sample. 20 mg of the dried sample was extracted with 2 mL of KOH/MeOH (1g/4mL) for 8 hours at 68°C with agitation in the middle of the incubation time and at the end. All measurements were performed in duplicates.

The ethylation of methylmercury occurred after the addition of lactic acid (4M) in 30 mL of NANOpure® water. Under these conditions an optimal pH of 4,7 is needed for ethylation.

The volumes of the sample and the lactic acid used varied between 25 to 1000 µL.

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The ethylated methylmercury produced was then transferred in a Tenax® column with 5 minutes agitation and a 10 minute bubbling with compressed air.

The column was dried with air for 10 minutes before being fixed to a Gas Phase Chromatography (argon flux 15 mL/min, column heat at 75°C). The column was composed of 15% OV3 chromosorb WAWDMCS 60/80 mesh.

Methylmercury was thermally desorbed from Tenax® by heating for 1 minute. At the Retention Time of 6 minutes it was quantified by atomic absorption.

A series of analysis always included blanks and replicates of the certified standard IAEA 405. The instrument was calibrated by ethylation of a known amount of MeHgCl. The detection limit of this method is 0,05 ng Hg/g for a 20 mg sample. This value corresponds to 3 times the blank variability. The quantification limit corresponding to 10 standard deviations was 0,15 ng Hg/g.

The accuracy of the method was evaluated by the analysis of the certified standard IAEA 405 which is sediment coming from the International Atomic Energy Agency. The certified value was 5,49± 0,53 ng.Hg/g.

6.4 Method to measure total mercury in planktonic samples

These measurements were performed in Bordeaux (EPOC laboratory by Régine Maury-Brachet's Team) and were applied to the planktonic samples.

Total mercury concentrations were determined by flameless atomic absorption spectrometry. Analyses were carried out automatically after drying the sample by thermal decomposition at 750 °C, under an oxygen flow (AMA 254, Leco-France).

The accuracy of total Hg determinations for each analytical batch was determined by the use of three certified reference materials: TORT-2, lobster hepatopancreas; DORM-2, dog-fish muscle; and DOLT-2, dogfish liver, from the National Research Council of Canada. Values determined were consistently within the certified ranges. Method precision of total Hg determinations, estimated from five replicates was 5%. All biota concentrations were reported on a dry weight basis (45 °C over 2 days).

Once the methods presented, the following chapters (from 4 to 11) will present the different results obtained during the PhD.

Chapter 4: Micro organisms isolated from snow

1 Introduction of the chapter

This chapter presented the micro organisms isolated from snow. It represents the beginning of the work on this subject.

The chapter is divided in two parts, the first one presents the bacteria isolated from Ny-Ålesund snow under the form of the paper : "Bacterial characterization of the snow cover at Spitzberg ,Svalbard" by Amato, Hennebelle et al, 2007". On this paper my participation was to have performed the biodegradation experiment and helped during the writing process.

The results presented in this paper suggests that snow contains bacteria able to degrade organic acids so to live in this environment, especially during the melted period. Therefore they could play a role in mercury cycle in arctic environment.

The second part of this chapter presents the yeast also isolated from snow.

The results obtained constituted the basis of our work about micro-organisms in the snow.

2 Bacterial characterization of the snow cover at Spitzberg, Svalbard

Bacterial characterization of the snow cover at Spitzberg, Svalbard

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Editor: Rosa Margesin

Keywords
bacteria; snow; Arctic; cold environment; biodegradation.

Abstract

A sampling campaign was organized during spring 2004 in Spitzberg, Svalbard, in the area around the scientific base of Ny-Ålesund, to characterize the snow pack bacterial population. Total bacteria counts were established by 4′,6-diamino-2-phenylindole (DAPI) in the seasonal snow pack bordering the sea. On the sea shore, bacterial concentration was about 6×10^4 cells mL^{-1}, without any significant variation according to depth. In the accumulation snow layer of the glacier, concentrations were about 2×10^4 cells mL^{-1}, except in the 2003 summer layer, where it reached 2×10^5 cells mL^{-1}, as the result of cell multiplication allowed by higher temperature and snow melting. Strains isolated from the seasonal snow pack were identified from their 16S rRNA gene sequences, and lodged in GenBank. They belong to the *Alphaproteobacteria*, *Betaproteobacteria* and *Gammaproteobacteria*, *Firmicutes* and *Actinobacteria*. They are closely related to cold environment bacteria, as revealed by phylogenetic tree constructions, and two appear to be of unknown affiliation. Using ^1H nuclear magnetic resonance, it was shown that these isolates have the capacity to degrade organic compounds found in Arctic snow (propionate, acetate and formate), and this can allow them to develop when snow melts, and thus to be actively involved in snow chemistry.

Introduction

Cold environments represent a large part of the Earth's biosphere, and their microbiota are of increasing interest. These can provide habitats for cells (Psenner & Sattler, 1998; Sattler *et al.*, 2001) that are already strongly suspected to play chemical roles in glaciers (Skidmore *et al.*, 2005), atmospheric clouds (Amato *et al.*, 2005) and more largely in water environments of these cold regions (Margesin & Schinner, 2001; Price & Sowers, 2004). It has been shown that bacterial activity can occur at subzero temperatures (Carpenter *et al.*, 2000; Junge *et al.*, 2004) and several physiological characteristics can allow such species to be active, for example spore formation, pigmentation (Fong *et al.*, 2001; Mueller *et al.*, 2005), increase in membrane fluidity (Seshu Kumar *et al.*, 2002) and production of enzymes active at low temperature (Groudieva *et al.*, 2004). The bacterial genera most commonly found in these cold environments belong to, in descending order of abundance, the *Proteobacteria* (especially *Alphaproteobacteria*, *Betaproteobacteria* and *Gammaproteobacteria*), *Cytophaga–Flavobacterium–Bacteroides* group, and low- and high-G+C Gram-positive genera (references above, and Christner *et al.*, 2001; Zhang *et al.*, 2002; Miteva & Brenchley, 2005).

Over the past few years, many studies have enabled the microbial content of various polar or mountain cold environments to be characterized, and the highest bacterial abundance values observed were reported from Arctic sea ice, where concentrations can reach 1×10^5 bacteria mL^{-1} of melted ice (Brinkmeyer *et al.*, 2003; Junge *et al.*, 2004). Fewer than 3×10^3 bacteria mL^{-1} were detected in the deep accretion ice of the subglacial Antarctic lake Vostok (*c.* 3500 m below the surface) (Karl *et al.*, 1999; Priscu *et al.*, 1999; Abyzov *et al.*, 2001). Intermediate bacterial concentration values were obtained for snow from glaciers (Skidmore *et al.*, 2000; Zhang *et al.*, 2002; Foght *et al.*, 2004), polar and alpine lakes (Alfreider *et al.*, 1996; Pearce *et al.*, 2003), and clouds (Bauer *et al.*, 2002; Amato *et al.*, 2005). For mid-latitude environments, mountain snow bacterial concentrations have been reported to range from 3×10^3 cells mL^{-1} (Bauer *et al.*, 2002) to *c.* 4×10^5 cells mL^{-1} (Alfreider *et al.*, 1996; Sattler *et al.*, 2001; Segawa *et al.*, 2005). However, the bacterial content of surface snow in polar regions has not thus far been investigated. Here we present the

microbiological characteristics of a seasonal snow pack from Ny-Ålesund (Spitzberg) with (1) its bacterial load, (2) the identification of cultivable bacteria and (3) their ability to degrade organic substances present in Arctic snow.

Materials and methods

Sampling sites

Samples were collected from two sites: the snow cover in the vicinity of the scientific base of Ny-Ålesund (78°56′N, 11°52′E, 10 m a.s.l., Spitzberg, see Fig. 1), and in the accumulation area of the Kongsvegen glacier (78°45′N, 13°19′E, 670 m a.s.l.), about 40 km east of Ny-Ålesund. The Ny-Ålesund site is located by the sea, along the south coast of the Kongsford, which is orientated east-west and open to the sea on its west side. The glacier is of north-west/south-east orientation, in the continuity of the Konsfjord.

Sampling method

All samples were taken with great care in order to avoid contamination, by wearing sterile gloves and a mask and using sterile material wrapped in two successive bags that had been sterilized by autoclaving. To collect snow, the surface in contact with the air was first systematically scratched using a sterile spoon and discarded. A sterile tube (50 mL) was then embedded into the snow to fill it without any need for extra manipulation, which could provide a source of contamination. Samples from different depths were taken at the two sites to establish total cell profiles, from pits dug into the Ny-Ålesund seasonal snow cover and in the annual layer of the Kongsvegen glacier (i.e. 170 cm deep). At each sampled depth, at least three samples were taken and two pits were investigated at each of the two sites. In addition, samples for cultures were collected on the day prior to our departure from the Ny-Ålesund site, at *c.* 3–15 cm snow depth, using the same protocol.

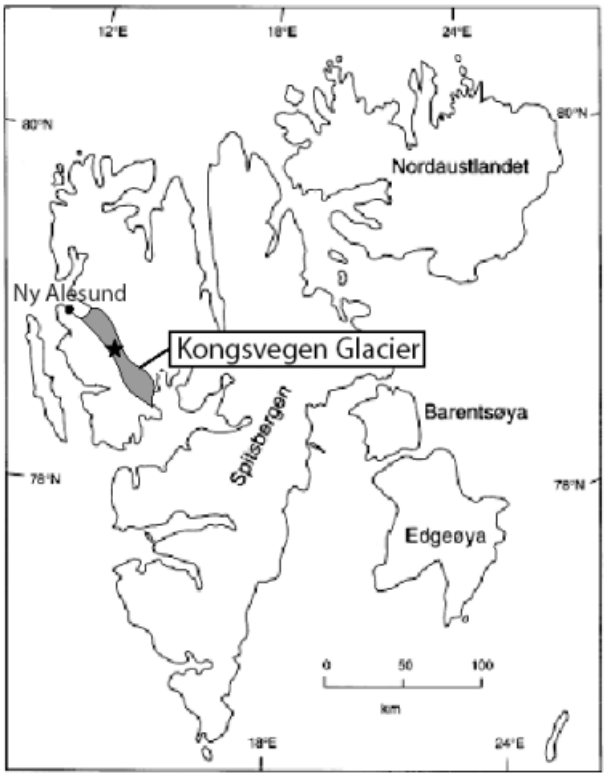

Fig. 1. Map of Spitzberg showing the locations of Ny-Ålesund and the Kongsvegen glacier.

Radionuclide measurements

To characterize the annual accumulation layer of the Kongsvegen glacier, radio nuclides were measured in snow samples collected for that purpose, just after those taken for microbial investigations.

Using a method developed by Delmas & Pourchet (1977), snow samples were melted (~500 mL, every 10 cm of snow equivalent), weighed, acidified and filtered on ion exchange paper, where all radionuclides were trapped. After drying, the filters were directly analysed by γ-spectrometry using a low-background germanium detector (germanium diode N type) (Pinglot & Pourchet, 1994). Because of low activity readings, some samples were combined for radioactivity analysis. For high-resolution gamma spectrometry, the analyser was protected against all interfering ambient radioactivity, in particular using an anti-Compton device. This system provides a lower detection threshold, especially for the isotopes of interest, such as ^{210}Pb (22.3-year half-life) and ^{7}Be (53 days) (Pinglot & Pourchet, 1994; Pourchet *et al.*, 2003). Standard ^{137}Cs, ^{210}Pb and ^{241}Am liquids from the CEA or Amersham laboratories (2% uncertainty at 95% confidence level) were used to calibrate the detector. The analytical procedures were the same as those used for the snow samples. The ^{210}Pb and ^{7}Be measurements were carried out with a time resolution between 24 and 72 h. Quantitative analysis software (GENIE 2000 v1.4; Canberra) was used to compute the activity of existing radionuclides and the associated accuracies. The total uncertainties due to both sampling procedures and counting statistics are of the order of 20% for ^{210}Pb and 50% for ^{7}Be. The ^{7}Be-specific activities were corrected for decay to the deposition time and counting. Blank and background values were checked regularly.

Total bacteria counts

Samples for total cell counts were treated immediately following collection. They were slowly melted at ambient temperature (about 15 °C) and fixed with an equal volume of prefiltered (0.22 μm; Millipore) 4–5% formaldehyde, and

stored at 4 °C before analysis. Each of the 30-mL fixed samples was incubated for 20 min in the dark with 2.5 μg mL^{-1} (final concentration) of 4′,6-diamino-2-phenylindole (DAPI) to stain cells (Porter & Feig, 1980), filtered (dark GTPB filters of 0.22 μm pore size) and rinsed with prefiltered (0.22 μm) distilled water. Filters were then mounted on slides, observed under epifluorescence microscopy (Olympus BH-2), × 1000 magnification, and cell numbers present on random fields were counted (at least 30 fields). Standard errors were calculated from at least three samples, from each of the two pits. Blanks, comprising filtered and distilled water only, were regularly checked.

Cultures and isolations of bacteria

Each sample to be used for cultivation was stored and transported frozen from Ny-Ålesund to the laboratory in France for analysis. It was then slowly and completely melted at ambient temperature, and divided to be incubated under different conditions. First, triplicates of 0.1 mL were directly plated onto solid R2A (Reasoner & Geldreich, 1983; Difco), and trypcase soy (TS) (bioMérieux) and Sabouraud (Difco) media supplemented with agar (20 g L^{-1}), to be incubated at 4, 15 and 27 °C. By contrast, liquid cultures were performed by enrichment of the melted snow with TS (1% and 50% v/v final concentration of the nutritive medium initially prepared at 30 g L^{-1}) and R2 (1% and 50% v/v final concentration of the nutritive medium initially prepared at 3.2 g L^{-1}) media. Liquid R2 was prepared according to the recipe of the commercial medium R2A, but free of agar. Triplicates of 100 mL of all these four media were made in 500-mL flasks incubated at 17 °C, under agitation (200 r.p.m.). When growth was visually detected, 0.1 mL was plated onto the corresponding solid medium for isolation of colonies. Each colony was differentiated from others on the basis of morphological criteria, and isolations were ensured by successive transfers onto the same medium if necessary. For all the experiments, prechilled filter tips and media were used to preserve cells from any heat shock.

Phylogenetic analysis and tree construction

Cell pellets obtained after centrifugation of liquid pure cultures of the isolated strains were resuspended into phosphate-buffered saline (PBS) solution and their total genomic DNA was extracted using the Easy DNA Kit (Invitrogen). Extracts were verified by gel electrophoresis, and 16S rRNA genes were amplified by PCR. This step was carried out using universal primers for *Eubacteria*: F8-Eub (5′-AGA GTTTGATCMTGGCTC-3′) and 1492r-Univ (5′-GNTACCTTGTTACGACTT-3′) (Humayoun *et al.*, 2003), in which M corresponds to A or C, and N to any one of the four nucleotides. About 100 ng of genomic DNA and 1.5 U of *Taq* polymerase (QBiogene) were employed. PCR was performed as follows: 25 cycles of 30 s at 94 °C for DNA, 30 s at 55 °C and 90 s at 72 °C, preceded by 5 min at 94 °C and ending with 7 min at 72 °C. PCR products were checked by gel electrophoresis, and purified using a Strataprep purification kit (Stratagene). They were finally freeze dried and sequenced by capillary electrophoresis (MWG-Biotech), using the previously described F8-Eub and 1492r-Univ primers, and a central primer, F500 (5′-CTAACT ACGTGC-CAGCAGC-3′), in order to obtain overlapping sequences. Comparisons of almost complete reconstructed 16S rRNA gene sequences with those included in GenBank were performed using the BLASTN interface, available at http://www.ncbi.nlm.nih.gov/BLAST/.

Multiple alignments of the 16S rRNA gene sequences obtained with some selected from among the closest related neighbours and others selected in the literature were achieved using CLUSTALW (http://www.ebi.ac.uk/clustalw/) and visually checked. Lengths of sequences were adjusted according to these multiple alignments and final lengths of 1367 and 1386 bp were thus compared for Gram-negative and Gram-positive isolates, respectively. One hundred trees generated by bootstrap were analysed by the neighbour-joining method of the PHYLIP 3.63 package (Felsenstein, 2004), after application of the Kimura two-parameter correction on calculated distances (correction of differences between rates of tranversion and transition) (Kimura, 1980). Two consensus trees were finally constructed to place both our Gram-negative and Gram-positive isolates.

Metabolic activity and ^{1}H NMR analyses

For each isolated strain tested, liquid precultures were first made at 17 °C in R2 broth for 48 h. Then, cultures were made under the same conditions, by transferring 3% v/v of preculture into fresh R2 broths. After 24–48 h growth, cells were harvested by centrifugation (4000 ***g***, 15 min, 4 °C), rinsed twice with NaCl 0.8%, and resuspended in test medium in order to obtain a final $OD_{580\,nm}$ of 10. The test medium was composed as follows: 25 mL of 0.1 M phosphate buffer adjusted to pH 7.0, containing 20 mM of propionate (C_3), acetate (C_2) or formate (C_1). Blanks, constructed by incubation of cells without added substance, were used to check that no compound was obtained from endogenous cell metabolism taking place in the incubation media.

For analysis, supernatants were separated from cells by centrifugation (12 000 ***g***, 3 min), and analysed by ^{1}H nuclear magnetic resonance (NMR) to evaluate biodegradation abilities of each strain for each of the three compounds (Grivet *et al.*, 2003). NMR samples were prepared as follows. Supernatants (450 μL) from biodegradation tests were supplemented with 50 μL of a 20 mM solution of $TSPd_4$ (sodium tetra deuteriated trimethylsilyl propionate;

Eurisotop) in D_2O (Eurisotop). D_2O was used for locking and shimming while $TSPd_4$ constituted a reference for chemical shifts (0 p.p.m.) and quantification. ^1H NMR spectra were recorded at 400.13 MHz on a Bruker Avance 400 spectrometer (Bruker) at 21 °C with 5-mm-diameter tubes containing 500 μL of sample. Thirty-two scans were collected (90°pulse, 4789.27 Hz SW, 655 36 data points, 6.84 min total acquisition time for one spectrum). Water signal was eliminated by presaturation. No filter was applied before Fourier transformation but a baseline correction was performed on spectra before integration with Bruker software. Under these conditions, the limit of quantification was in the range 0.05 mM. The concentration of metabolites was calculated as follows: $[m] = (9 \times A_0 \times [TSPd_4])/(b \times A_{ref})$, where $[m]$ is the concentration of metabolite, A_0 is the area of metabolite m resonance, A_{ref} is the area of reference resonance in the ^1H NMR spectrum, b is the number of protons of metabolite m in the signal integrated, and 9 is the number of protons resonating at 0 p.p.m.

For each of the three substrates, percentages of biodegradation over a period of 24 h were calculated from concentrations measured in the incubation media (supernatants) at $t_{0\,h}$ and $t_{24\,h}$ (respectively, $Ct_{0\,h}$ and $Ct_{24\,h}$), as follows: $[(Ct_{0\,h} - Ct_{24\,h})/Ct_{0\,h}] \times 100$.

Results and discussion

Bacterial concentration profiles and seasonal snow characteristics

Bacterial counts made all along the snow cover are shown in Fig. 2(a). The total snow depth at Ny-Ålesund was about 70 cm, and bacterial concentration was homogeneous at about 6×10^4 cells mL^{-1} over this depth. This concentration is about one order of magnitude lower than those found in sea ice (Brinkmeyer *et al.*, 2003), but slightly higher than the concentration measured in the snow cover of Alpine sites (1.1×10^4 cells mL^{-1} in Sattler *et al.*, 2001; 2×10^4 cells mL^{-1} in Bauer *et al.*, 2002).

The snow pack studied here corresponded to snow deposited from October/November 2003 to early spring 2004. The determination of bacterial abundance in a 200-cm profile (Fig. 2b) on the Kongsvegen glacier allowed us to determine the variability of bacterial snow content for 1 year's snow precipitation, given that 170 cm represented the snow deposits accumulated since the end of summer 2003 (i.e. the 2003–2004 period). Specific activity of ^7Be clearly showed the presence of two major types of snow [noted as snow (a) and snow (b)] along this annual profile. In the upper layer [e.g. snow (a)], from the surface to a depth of 80 cm, mean ^7Be activity ranged from around 242 to 920 mBq kg^{-1}. Deeper, from 80 to 165 cm, the low activity, reaching no more than 120 mBq kg^{-1}, supports a clear

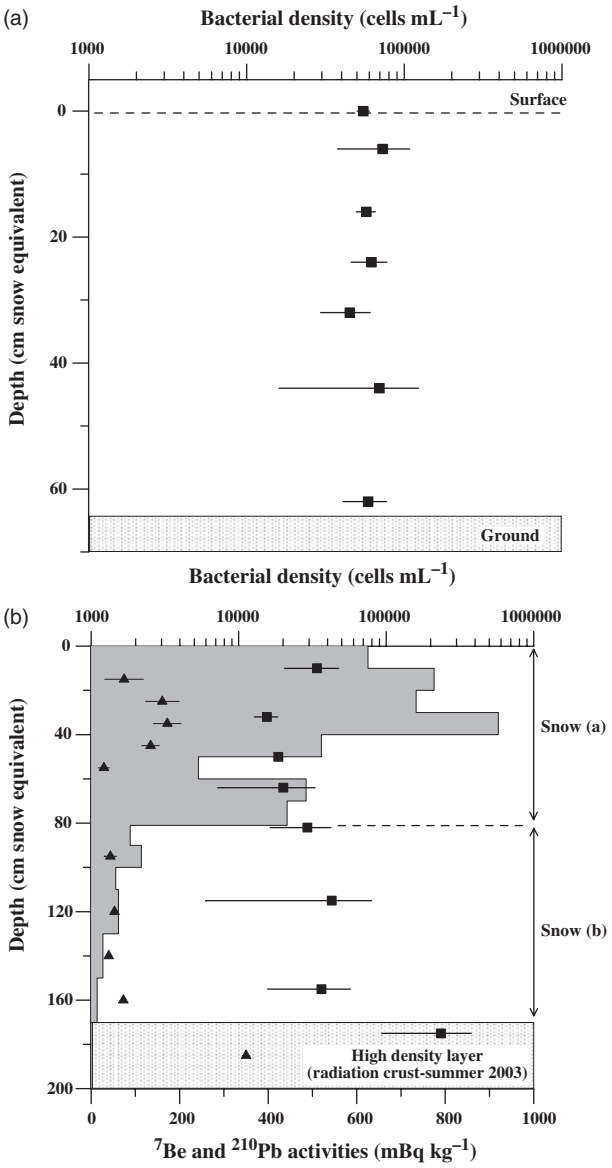

Fig. 2. Bacterial concentration (■) expressed as the number of cells mL^{-1} of melted sample, in the seasonal snow cover of the Ny-Ålesund site (a) and along the Kongsvegen glacier (b). Radionuclide-specific activities of ^7Be (shaded area) and ^{210}Pb (▲) were also measured along the annual accumulation snow layer of the glacier: snow (a) corresponds to fresh snow, deposited in winter (< 3 months), and snow (b) to that deposited between summer and winter; the deeper high-density layer (radiation crust) was the surface of the glacier during summer 2003.

difference of age between the two layers. Owing to radioactive decay properties and taking into account the variability of ^7Be atmospheric concentrations in this area, snow (a) was expected to be < 3 months old, and snow (b) corresponded to the period evaluated between the end of summer 2003 and January/February 2004. Below snow layer

Table 1. Isolated strains: conditions of isolation and description

Strain	Identification (sequence similarity)	GenBank accession number	Cell morphology	Colony pigmentation and morphology	Isolation temperature (°C)	Isolation media
A	*Chelatococcus* sp. (98.6%)	DQ497243	Short rods	Yellow, mucoid	27	Direct isolation, R2A
C	*Brevundimonas* sp. (99.5%)	DQ497234	Long, small thin rods	Orange, mucoid	17	Isolation after enrichment, 1% TS; 50% R2
D	*Bacillus* sp. (100%)	DQ497235	Thick rods	Translucent white, mucoid	17	Isolation after enrichment, 50% R2
E	*Micromonospora* sp. (99.7%)	DQ497236	Mycelium	Orange-red, raised, folded	17	Isolation after enrichment, 1% R2
H	*Hydrogenophaga* sp. (99.6%)	DQ497237	Small rods	Cream, mucoid	17	Isolation after enrichment, 50% TS; 1% TS
I	*Moraxella* sp. (99.7%)	DQ497238	Small rods	Translucent white, mucoid	17	Isolation after enrichment, 1% TS
L	Undetermined *Sphingomonadaceae* (95.2% with *Sandarakinorhabdus limnophila* AY902680)	DQ497240	Small rods	Orange, mucoid	17	Isolation after enrichment, 50% R2; 1% R2
N	Undetermined *Sphingomonadaceae* (94.5% with *Sandarakinorhabdus limnophila* AY902680)	DQ497241	Small rods	Orange-yellow, mucoid	17	Isolation after enrichment, 50% R2
P	*Paenibacillus* sp. (100%)	DQ497239	Very thick rods	White, matt	17	Isolation after enrichment, 1% R2
R	*Agromyces* sp. (100%)	DQ497242	Rods and mycelium	Pale yellow, mucoid	17	Direct isolation, TSA

Accession numbers of the 16S rRNA gene sequences submitted to GenBank are given. Strains are designated by nonconsecutive letters owing to redundancies of isolations in several cases.

(b), we found snow deposits from the autumn 2002 to summer 2003 period. This assumption was attested by:

(1) The nearly complete depletion of ^{7}Be activity below a depth of 170 cm. Indeed, we know that over 5–6 half-lives, more than 98% of the initial concentration of a radioactive element is depleted. In our case, because of the half-life of ^{7}Be fallout (53.4 days), 5–6 half-lives correspond to 250–300 days, i.e. about '1 year's' accumulation as previously defined. So, the nondetection of ^{7}Be activity below 170 cm indicated than '1 year' had been exceeded.

(2) The increase in snow density (not shown in Fig. 2) and of ^{210}Pb activity in the snow profile below 170 cm depth. These factors could result from metamorphism (i.e. the melting process during summer 2003) of 2002–2003 snow deposits, as attested to by variations in snow grain sizes and structure in this snow layer, defined as the radiation crust. As a consequence of this metamorphism, chemical species, such as ^{210}Pb, were concentrated in the resulting denser layer, as observed in Fig. 2(b).

The bacterial concentration varied from 1×10^4 to 4×10^4 cells mL^{-1} in the upper snow layers (Fig. 2b). These values are in the same range as those measured by Bauer *et al.* (2002) and by Segawa *et al.* (2005) in high mountain snow covers at midlatitude. This recorded concentration is consistent with the bacterial concentration found in the seasonal snow pack (Ny-Ålesund site), and it can thus be estimated that snow deposition in this region leads to a constant bacterial abundance in snow. The count performed in the radiation crust, corresponding to the surface of the glacier during summer, reached a significantly higher value of 2×10^5 cells mL^{-1} (Student's t-test, $P < 0.01$). It represents the previously explained melting/percolation processes, and could also result from cell multiplication occurring on the surface of the glacier during summer.

Isolated strains

Ten distinct bacterial strains were recovered by cultivation and successfully identified. Table 1 details the culture media in which they were detected, and the macroscopic characteristics of the colonies (pigmentation and morphology) once transferred onto solid media.

After about 1 month of cultivation, direct plating of melted snow on agar media only led to the recovery of two strains A and R, growing sparsely at 27 °C on R2A and at 17 °C on TS agar, respectively. At 4 °C and after 2 months of cultivation, only a single fungal colony was observed on R2A medium (not shown here) but no bacterial growth in any media was detected. As a consequence of the very low recovery of cells by direct plating, counts of cultivable cells were not performed. By contrast, enrichments of melted snow with nutrients (TS or R2A compounds) incubated at 17 °C provided conditions for growth for many more bacterial strains. Indeed, eight strains were recovered by this

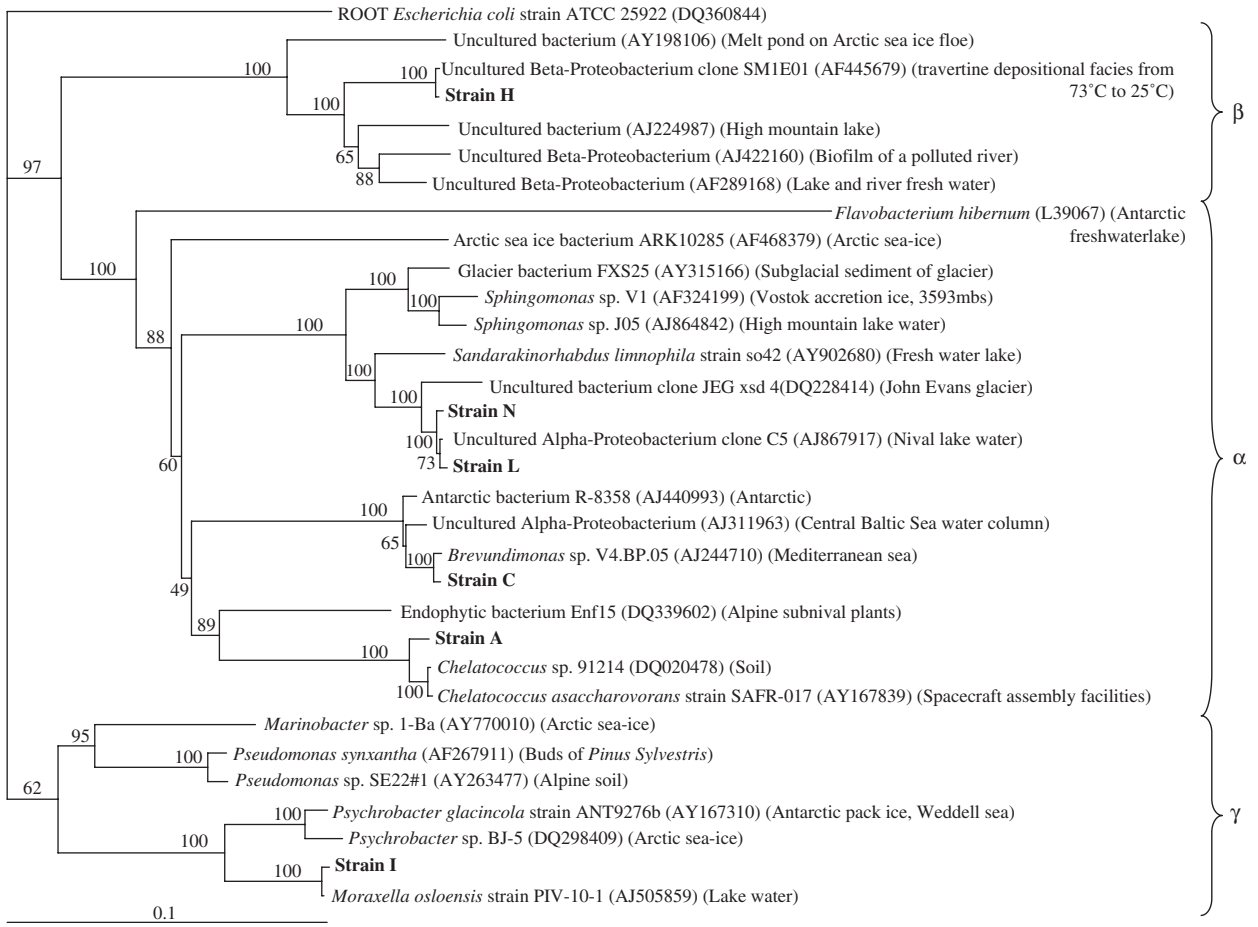

Fig. 3. Phylogenetic analysis of the 16S rRNA genes for the Gram-negative isolates and selected strains. The tree was generated by the neighbour-joining method after alignment of 1367 bp, and rooted with *Escherichia coli* ATCC 25922. Bootstrap values (100 replications) are specified for each node, and GenBank accession numbers and isolation sources are provided in parentheses. Scale bar indicates 0.1 substitutions per nucleotide.

method: one from flasks containing snow enriched with 50% of TS (TS 50%) (H), three from TS 1% (C, H and I), four from R2 50% (C, D, L and N) and three from R2 1% (E, L and P). In our case, these results suggest that the latter method is more appropriate to investigate the cultivable fraction that can be found in polar snow. This was directly inspired from works of Skidmore *et al.* (2000) and Bussmann *et al.* (2001). These authors have already successfully used such methods of amendment. Our incubation conditions allowed the isolation of strains able to develop under low nutrient conditions and at relatively low temperature, meaning they were oligotrophic and at least psychrotolerant microorganisms.

Cells of the strains were all rod shaped, except E, which was filamentous. More than the half of the isolated strains formed pigmented colonies. Four of them presented an orange pigmentation and two were yellow; others were paler, from cream to translucent white. Pigmented strains are frequently found at high proportions in such cold environments (Foght *et al.*, 2004; Mueller *et al.*, 2005). The presence of pigments has been suggested to play a role in maintaining membrane fluidity as an adaptation to life at low temperature (Fong *et al.*, 2001).

With regard to the taxonomy of the isolates, genera found were well distributed among the two Gram groups, with six strains belonging to the Gram-negative branch and four belonging to the Gram-positive branch. The phylogenetic trees constructed to determine their affiliations are shown in Figs 3 and 4. All the Gram-negative isolates belong to the *Proteobacteria*, of which the largest proportion (four out of six) belong to the *Alphaproteobacteria*. These isolates include the unambiguously ($>$ 99% homology) identified genus *Brevundimonas* (strain C), and uncertain genera in the cases of strains N and L. Their closest neighbours obtained by BLAST were found in a nival lake and in the John Evans glacier, attesting to their adaptation to cold environmental conditions. However, they show only poor homology (about 95%), with their closest recognized neighbour,

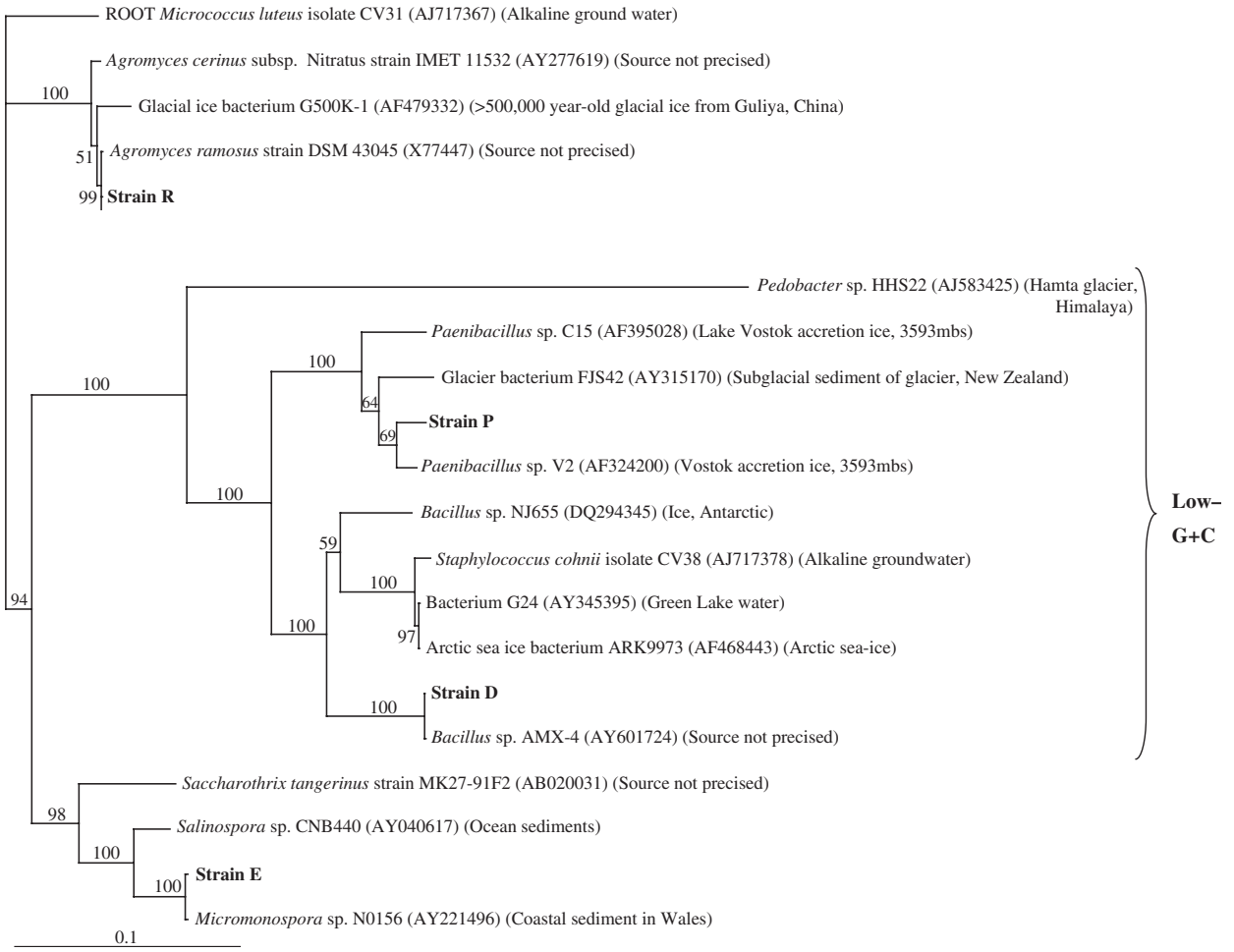

Fig. 4. Phylogenetic analysis of the 16S rRNA genes of the Gram-positive isolates and selected strains. The tree was generated by the neighbour-joining method after alignment of 1386 bp, and rooted with *Micrococcus luteus* CV31 from groundwater. Bootstrap values (100 replications) are specified for each node, and GenBank accession numbers and isolation sources are provided in parentheses. Scale bar indicates 0.1 substitutions per nucleotide.

Sandarakinorhabdus (Gich *et al*., 2005). They also show not more than 94% sequence homology with members of the genus *Sphingomonas*. As a consequence, these two strains are likely to be of as yet unknown affiliation. Strain C is more probably related to the sea, since it matches closely with isolates obtained from the Mediterranean and central Baltic seas. The 16S rRNA gene sequence of strain A shows $< 98.6\%$ homology with its nearest neighbours, *Chelatococcus* sp., found in soil and in the atmosphere (spacecraft). The *Betaproteobacteria* is also represented (strain H) by what appears to be a rare cultivated specimen of this affiliation, as shown by the phylogenetic tree: only uncultured bacteria are placed around strain H. This strain was identified as probably representing a species of the genus *Hydrogenophaga*. A member of the *Gammaproteobacteria* was cultivated (strain I), and identified as a species of the genus *Moraxella*, closely related to a strain isolated from lake water. Among the Gram-positive bacterial strains were two *Firmicutes* (low-G+C bacteria) and two *Actinobacteria* (high-G+C bacteria). The first are members of the genera *Bacillus* (strain D) and *Paenibacillus* (strain P), and the *Actinobacteria* are members of the genera *Micromonospora* (strain E) and *Agromyces* (strain R). Strains P and R appear to be related to bacteria found in deep ice cores, indicating their resistance to very cold conditions, while E is related to sea sediments, where the temperature generally encountered is around 4 °C.

All the genera we determined are well known to be widely spread in cold environments. *Alphaproteobacteria*, *Betaproteobacteria* and *Gammaproteobacteria* are abundantly found in pack ice at both poles, whether identified by cultivation or by molecular methods (Brinkmeyer *et al*., 2003; Groudieva *et al*., 2004). They are also among the dominant groups detected in the bacterial flora of the winter cover (snow and ice) and pelagic zone of a midlatitude high mountain lake (Alfreider *et al*., 1996). The *Betaproteobacteria*, in

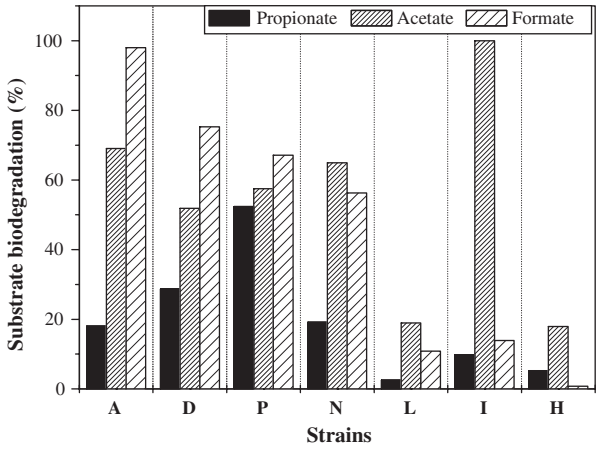

Fig. 5. Biodegradation abilities of the isolated strains on organic compounds at 17 °C. Concentrations measured by ^{1}H NMR were used to calculate the proportions of substrate biodegraded. Values are expressed as percentages of degraded compound after 24 h of incubation compared with the concentration measured at $t_{0\,h}$. Each strain was incubated with a single substrate at a time, in phosphate buffer at pH 7.0.

particular, was found to be highly dominant beneath several glaciers (Skidmore *et al.*, 2005). On the whole, Gram-negative bacteria are more often reported than Gram-positive bacteria from poles and glacier samples. Thus, Miteva & Brenchley (2005) isolated low- and high-G+C Gram-positive bacteria in an ice core, including members of the genus *Paenibacillus*, which was also detected in Lake Vostok accretion ice (Christner *et al.*, 2001). Finally, it can be noted that genera such as *Moraxella*, *Peanibacillus*, *Bacillus* and *Agromyces* were also found in cloud water samples at midlatitude during winter (P. Amato *et al.*, unpublished data).

Most of the studies performed on cold environments also report the presence of *Cytophaga–Flavobacterium* strains, but no cultivable specimen of this affiliation was detected in our snow sample.

The presence here in snow of strains closely related to others commonly found in cold environments testifies to their resistance to cold conditions, whether by their spore-forming ability (in the case of some Gram-positive bacteria) or by pigments related to membrane fluidity. Furthermore, their ability to develop under oligotrophic conditions and at moderate temperature suggest their activity when the snow melts, and so also their involvement in the chemical composition of runoff water.

Metabolic activity of the isolated strains

To characterize the isolated strains further, we investigated their metabolic potential with respect to organic compounds. Formate (C_1), acetate (C_2), and propionate (C_3) were chosen as they were found in Arctic snowfall (Toom-Sauntry & Barrie, 2002). Biodegradation of these compounds by seven of the isolated strains was monitored by ^{1}H NMR spectroscopy. Percentages of degraded substrates after 24 h of incubation at relatively moderate temperature (17 °C) are shown in Fig. 5. The results show that globally all the strains have good potential to metabolize the compounds tested.

Propionate is highly transformed (more than 20% of the total concentration) by the two low-G+C strains (D and P). For four strains (A, D, N, P), more than 50% of the acetate was metabolized in 24 h, and strain I appeared to be very efficient in degrading this compound, with no acetate remaining in its incubation medium. In the case of formate, strains A, D, P and N can be regarded as highly active, with between 55% (N) and 99% (A) of the initial amount being metabolized during the incubation period. More precisely, three types of responses were encountered among the tested strains under these laboratory experiments. Strains D, P and A had an increasing biodegradation capacity with decreasing carbonaceous chain length (propionate $<$ acetate $<$ formate). Others showed a preference for acetate, with relative variations concerning the degradation of formate. For strains N and L, formate was almost as well accepted as acetate, whereas in the case of strains I and H, almost exclusively acetate was transformed. Strain I seemed to be very specialized for the latter compound and strains L and H showed low activity ($<$ 20%) whatever the substrate considered.

On the whole, these results show that strains isolated from Arctic snow samples have the capacity to degrade organic compounds found there at moderate temperature. This suggests that bacteria present in snow are able to metabolize such substrates to develop and to sustain growth in this environment during melting periods. This is consistent with the cell multiplication suspected to occur on the summer layer of the glacier (as discussed previously). As a consequence, it demonstrates their capacity to be actively involved in the chemistry of the snow cover.

Conclusion

These investigations on the microbial content of the snow cover of an Arctic site, Spitzberg, performed in April 2004 show that, for a seasonal snow pack, the concentration of bacteria was constant with depth, at around 6×10^4 cells mL^{-1} of melted snow. A bacterial concentration of about 2×10^4 bacteria mL^{-1} was recorded along the annual accumulation layer of the Kongsvegen glacier. The concentration observed in the radiation crust, which constituted the glacier surface during the previous summer, was one order of magnitude higher. This certainly resulted from

percolation and bacterial growth on the snow surface during summer.

Direct plating and enrichment of snow samples with low levels of nutrients led to the isolation of ten strains, for which 16S rRNA gene sequences were lodged in GenBank. They were identified as belonging to the *Alphaproteobacteria* (one *Brevundimonas* sp., one *Chelatococcus* sp., and two *Sphingomonadaceaes* of unknown generic affiliation), *Betaproteobacteria* (one *Hydrogenophaga* sp.), *Gammaproteobacteria* (one *Moraxella* sp.), *Firmicutes* (one *Paenibacillus* sp. and one *Bacillus* sp.) and *Actinobacteria* (one *Agromyces* sp. and one *Micromonospora* sp.). Their physiological properties, such as spore-forming ability, pigmentation, and capacity to grow in low-nutrient media and at moderate temperature, would make them able to sustain growth and activity when snow melts in spring. This idea is also supported by their ability to metabolize organic compounds such as propionate, acetate or formate, which are found in Arctic snow, providing them with a substrate for growth. Such growth of microorganisms when snow is melting and during the following summer is consistent with cell counts made along the annual accumulation layer of the glacier.

Finally, the bacterial characterization of the snow cover in Svalbard reported here opens new perspectives concerning the potential role of microorganisms in the chemical reactions taking place in Arctic snow. First of all, they could be involved in carbon balance, and also participate in other geochemical cycles. For instance, it would be of interest to study the interaction of the strains isolated with divalent mercury, a toxic metal, so as to understand better the role of microbiology in snow towards this metal and thus understand whether bacteria can play a significant role in its cycle.

Acknowledgements

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These results show that two strains were not totally identified. In the rest of the manuscript, these strains will be cited as *Sandarakinorhabus limnophila* 94,5 % and *Sandarakinorhabus limnophila* 95,2 %.

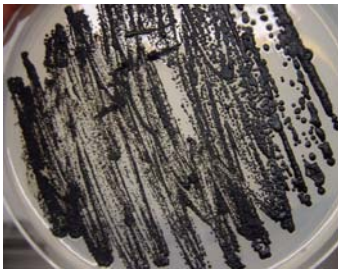
1 Yeasts isolated from snow

In the environment yeasts are also present and as they are eukaryotic organisms their behaviour could be different from prokaryotic bacteria.

In parallel to the bacteria, three yeasts strains were also isolated from Ny-Ålesund snow.

Their morphological aspect on R2 solid medium is presented by the following pictures.

- *Phaecoccomyces nigricans*.

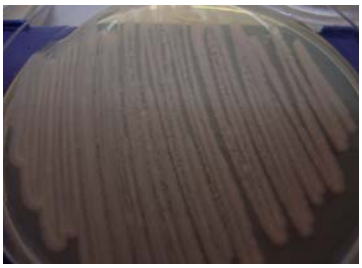


Picture 4. 1 : Colonies of *Phaecoccomyces nigricans* on solid R2 medium

- *Aureobasidium sp.1 (J)*

Picture 4. 2 : Colonies of *Aureobasidium sp1.* on solid R2 medium.

- *Aureobasidium sp 2.(G)*



Picture 4. 3 : Colonies of *Aureobasidium sp2* on solid R2 medium.

Phaecoccomyces nigricans is a rare strain which was only reported in a yeast collection of The University of Praha in Czech Republic.

Two strains were belonging to *Aureobasidium* species. These species had already been isolated from Arctic sea water and in glacier ice. (Gunde-Cimerman et al. 2003). The most common yeast of this genus is *Aureobasidium pullulans* which was known to be able to form exopolysaccharides (Lee et al. 1999). This property could be interesting if our strains also formed exopolysccharides because this kind of molecules was known to be capable to complex metals ions in the environment. This phenomenon could change the biodisponibility of metals and could protect micro organisms against metal toxicity. (Avery and Tobin 1993).

In any case in *Aureobasidium* genus, only *Aureobasidium pullulans* is well described so the two *Aureobasidium* species isolated from Ny-Alesund snow should be new species. Their identification was performed by CBS on the base of morphological criteria and they could not be identified as totally known species. *Aureobasidium sp2* was added by CBS to their library under the code CBS 123406.

Few data existed about yeast in polar environment, some strains were isolated from Antarctica's soil (Arthur et al in 1976) and also fungi (Fell, 2006) or from glacier's ice (Butinar et al. 2007). But no data was reported about yeast isolated from snow in Svalbard. This work indicated that such micro-organisms could be isolated from surface snow cover in this area.

2 Conclusion on micro organisms isolated from snow

These first results on microbial content in Ny-Ålesund snow showed the concentration of bacteria in the surface snow in 2004 was $6x10^4$ cells.$mL^{-1}$of melted snow.

Isolation and identification of the micro organisms led to the recovery of 10 bacterial strains belonging to genus usually found in cold environment (α-proteobacteria, β-proteobacteria, γ-proteobacteria, Firmicutes and Actinobacteria) and 3 yeasts (2 belonging to *Aureobasidium* species and one to *Phaecoccomyces* species).

Their ability, pigmentation, and capacity to grow in low-nutrient media and at moderate temperature, would make them able to sustain growth and activity when snow melts in spring.

This idea is also supported by bacterial ability to metabolize organic compounds such as propionate, acetate or formate, which are found in Arctic snow, indicating that during snowmelt growth could occur. This last idea was consistent with cell counts made along the annual accumulation layer of the glacier.

Once micro organisms isolated from snow their behaviour with temperature was investigated. Growth characteristics of such micro organisms were measured by testing their growth at 5, 12, 17 and 27°C.

The results obtained are presented in chapter 5.

Chapter 5 : Growth Characteristics of micro organisms isolated from snow

1 Introduction of the chapter

To better characterize the behaviour of the micro organisms toward temperature, each one was cultivated under several temperatures (5, 12, 17 and 27°C) in R2 medium under 200 rpm agitation. For each strains and any temperature the culture was started from the same preculture which were performed at 17°C because of laboratory constraint.

Once the growth curves obtained the optimal growth temperature was determined graphically.

The results obtained are presented in the following part.

2 Results

2.1 Bacteria

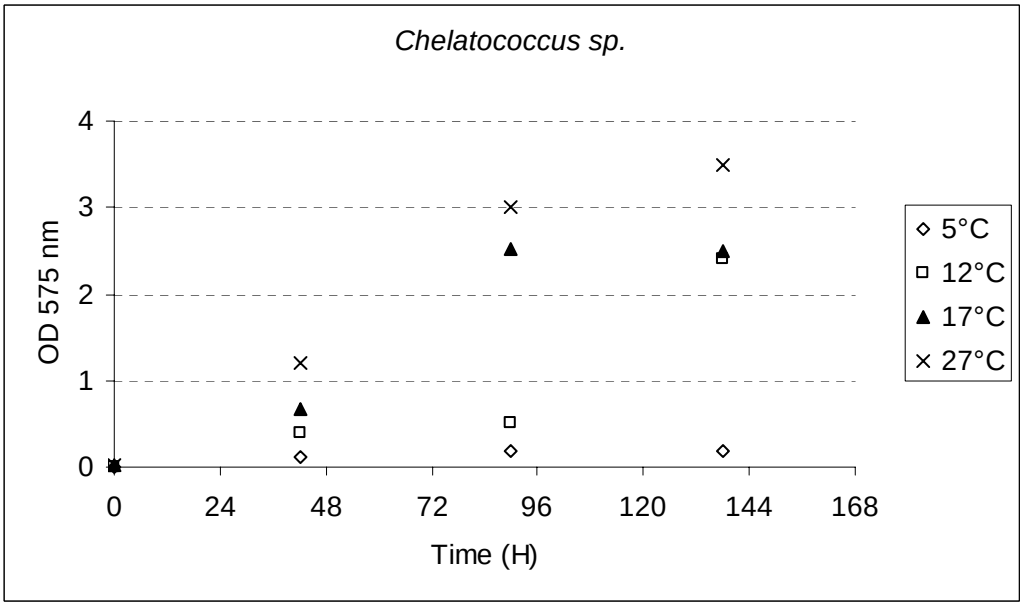


Figure 5.1 : Growth of *Chelatococcus sp.* at 5, 12, 17 and 27°C

Figure 5.1 presents the results obtained for *Chelatococcus sp.* This strain had an optimum growth temperature of 27°C. It also had a good growth at 17°C and needed an adaptation time at 12°C. No growth was detected at 5°C.

This strain had a behaviour close to a mesophilic bacteria but could grow at 12°C indicating that its minimum growth temperature was between 12 and 5°C.

So this strain can be classified as a mesophilic bacteria but with a good tolerance to colder temperature.

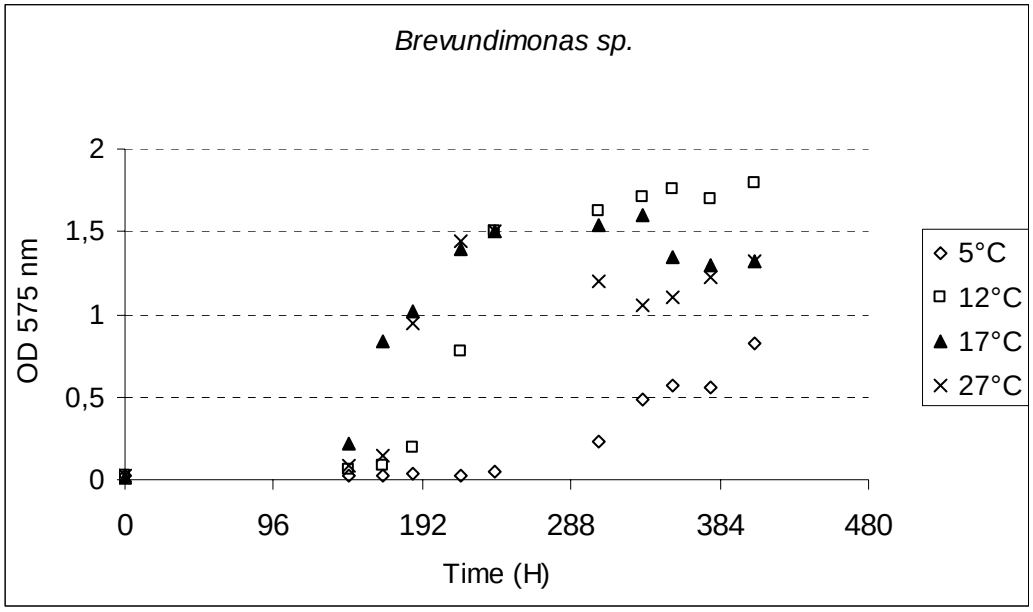


Figure 5. 2 : Growth of *Brevundimonas sp.* at 5, 12, 17 and 27°C

Figure 5.2 presents the results obtained for *Brevundimonas sp*.

The strain could grow at all the temperatures tested including 5°C.

For 27, 17 and 12°C the growth was globally the same with a longer Lag phase at 17°C probably resulting from the difference of temperature between preculture and culture.

Regarding these results, this strain could be consider as a psychrotroph microorganism.

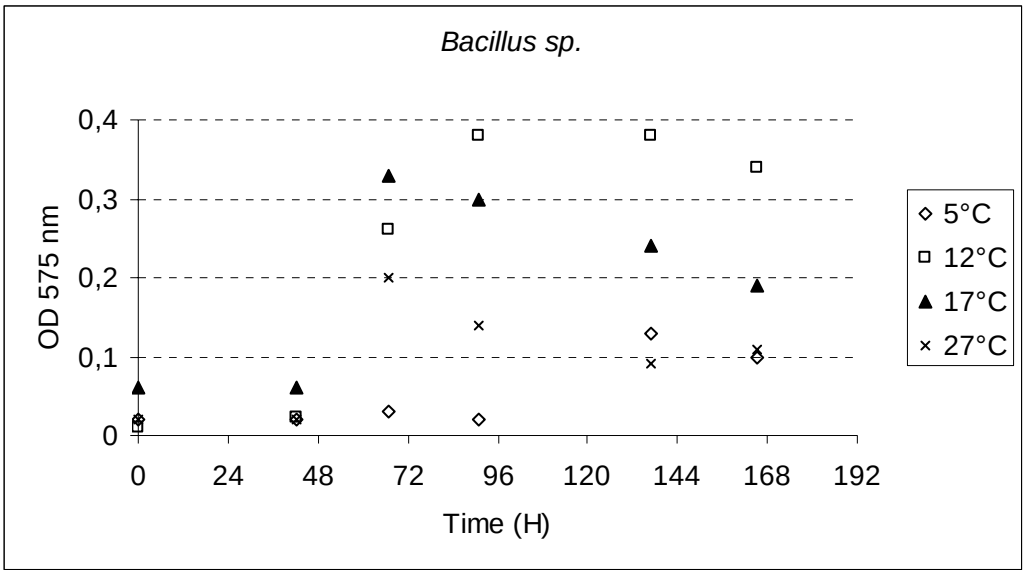


Figure 5. 3: Growth of *Bacillus sp.* at 5, 12, 17 and 27°C

Figure 5.3 presents the results obtained for *Bacillus sp.*

For this strain, the growth was globally low (OD maximum = 0,4) but the optimum growth temperature which gave higher OD result was 12°C. A growth with lower OD was observed at 17°C, and very low growth were observed at 5°C and 27°C (OD maximum = 0,14).

This strain has a behaviour close to a psychrophilic microorganism with an optimum growth temperature between 12 and 17°C .

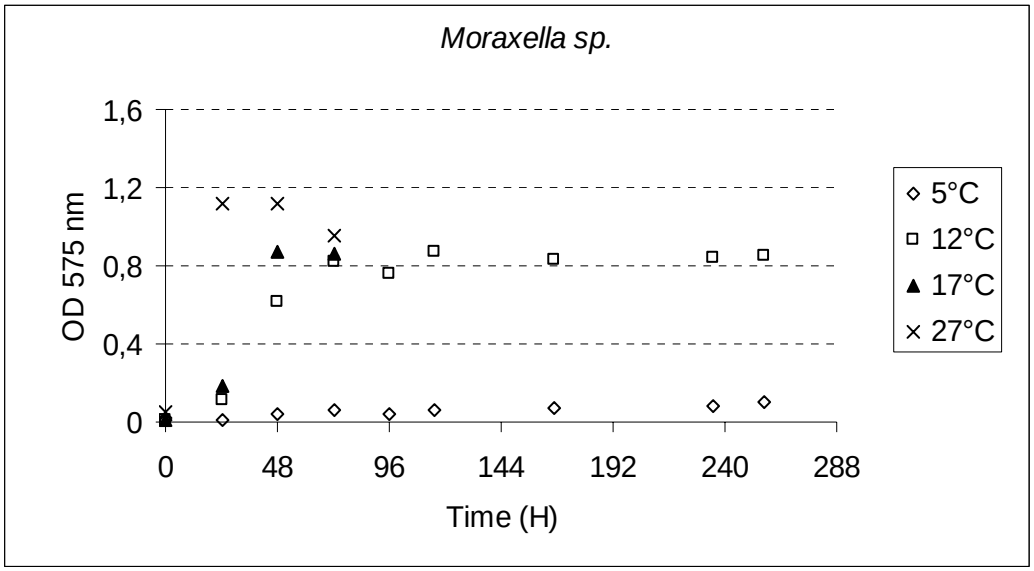


Figure 5. 4: Growth of *Moraxella sp.* at 5, 12, 17 and 27°C

Figure 5.4 presents the results obtained for *Moraxella sp.*

For this strain no Lag phase was observed at 27°C and the OD was maximum for this temperature (OD=1,18), this indicates that 27°C was its optimum growth temperature. The strain needed a little more time to grow at 17 and 12°C and gave lower OD results (OD = 0,8). Finally, no growth was observed at 5°C.

This strain could be considered as a mesophilic microorganism.

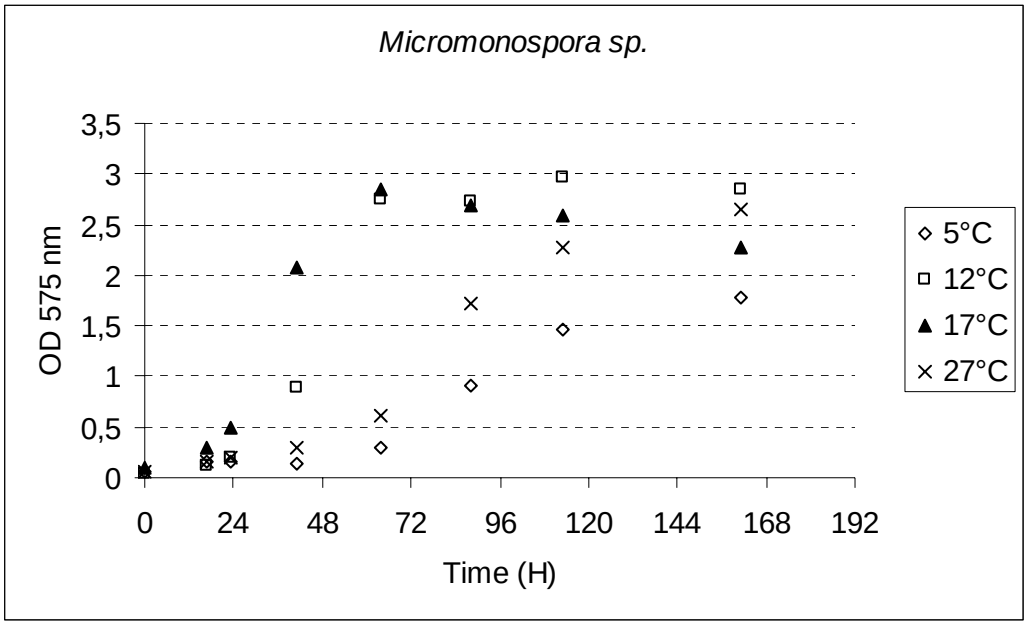


Figure 5. 5: Growth of *Micromonospora sp.*at 5, 12, 17 and 27°C

Figure 5.5 presents the results obtained for *Micromonospora* sp. This strain had comparable growth at 12 and 17°C. It was more disturbed at 27°C where it needed a longer Lag Phase.

Moreover *Micromonospora sp* was able to grow at 5°C.

These results indicated *Micromonospora sp.* has a behaviour close to a psychrotroph micro organism.

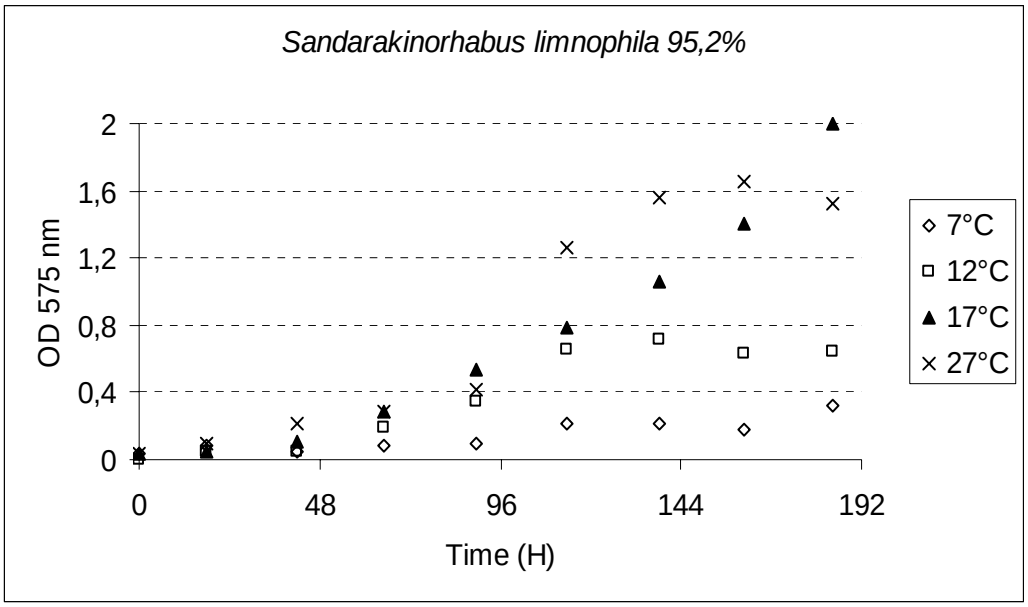


Figure 5. 6: Growth of *Sandarakinorhabus limnophila 95,2 %.* at 7, 12, 17 and 27°C

The results obtained for *Sandarakinorhabus limnophila 95,2 %* are presented in figure 5.6. This strain could grow slowly at every temperature (but very slowly at 7°C), the optimal growth temperature seemed to be 17°C.

Due to the optimal temperature of 17°C *Sandarakinorhabus limnophila 95,2 %* can be considered as a psychrotroph microorganism.

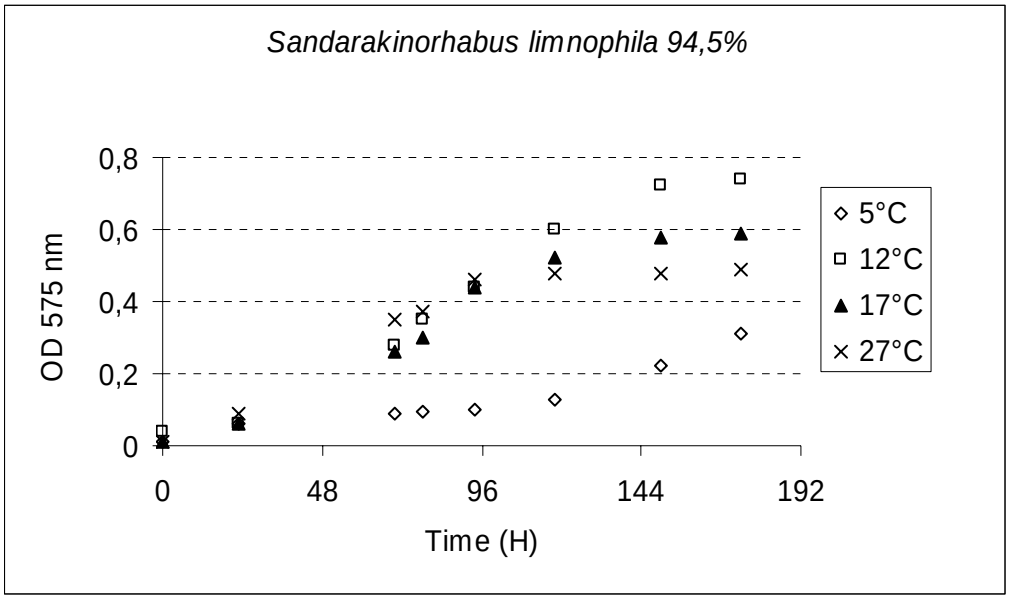


Figure 5. 7: Growth of *Sandarakinorhabus limnophila 94,5 %.* at 5, 12, 17 and 27°C

Figure 5.7 presents the results obtained for *Sandarakinorhabus limnophila 94,5%.* This strain had similar growth at 17 and 12°C, but at 27°C the growth gave lower OD. This strain was able to grow at 5°C with a longer Lag Phase.

Regarding the results, this strain can be considered as a psychrotroph micro organism.

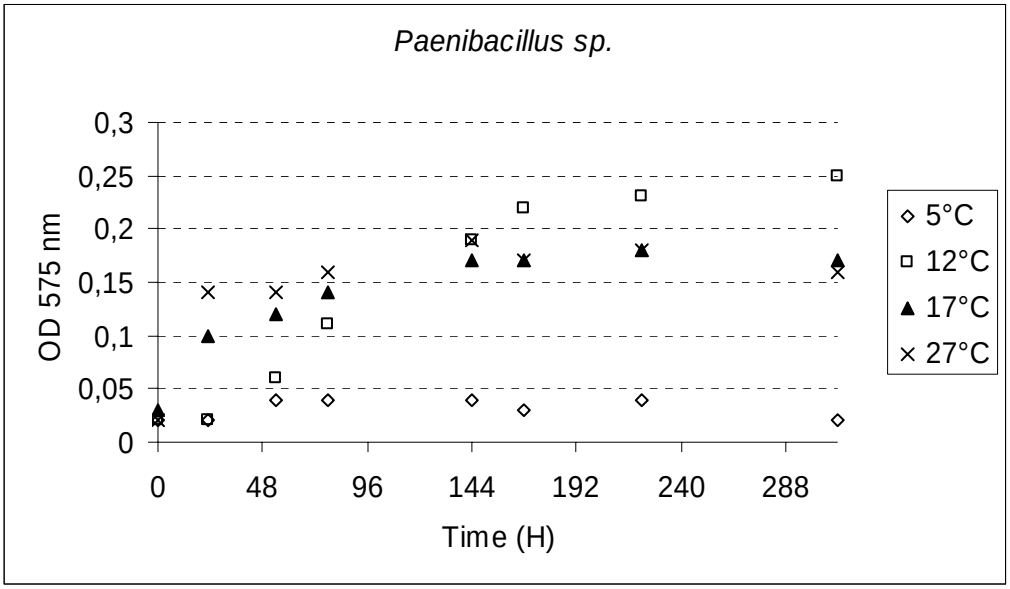


Figure 5. 8: Growth of *Paenibacillus sp.*at 5, 12, 17 and 27°C

Figure 5.8 presents the results obtained for *Paenibacillus sp.* This strain grew well at 27 and 17°C with no observable Lag phase. At 12°C the strain presents a lag phase but an OD max superior to that observed at 27 and 17°C.

This strain was not able to grow at 5°C.

These results indicates that *Paenibacillus sp*. has a behaviour close to a mesophilic microorganism.

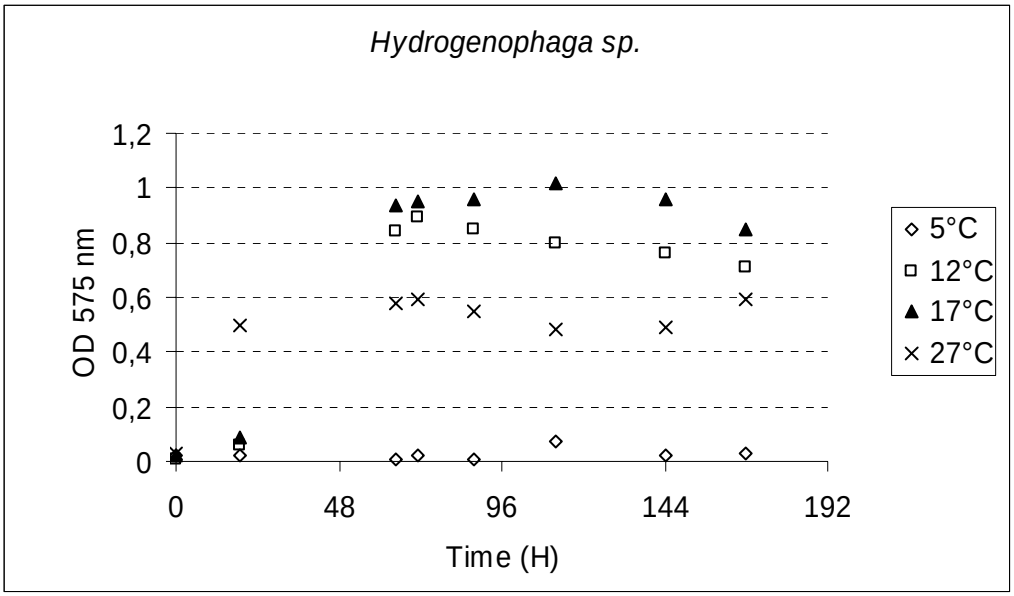


Figure 5. 9: Growth of *Hydrogenophaga sp.* at 5, 12, 17 and 27°C

Figure 5.9 presents the results obtained for *Hydrogenophaga sp.* This strain grew faster at 27°C but raised OD lower than the ones observed at 12 and 17°C. At these temperatures the growth was nearly the same. The strain was not able to grow at 5°C.

Regarding the fact that the strain was more disturbed at 27°C than for lower temperature. The strain could be classified as a psychrotroph micro organism.

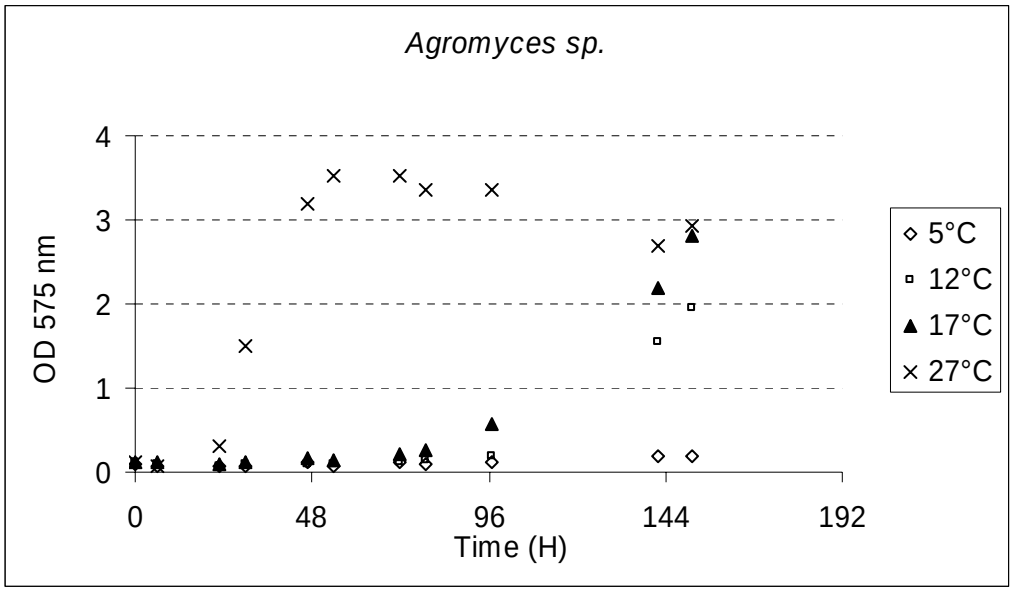


Figure 5. 10: Growth of *Agromyces sp.* at 5, 12, 17 and 27°C

Figure 5.10 presents the results obtained for *Agromyces sp*. The strain grew well only at 27 °C with a short Lag Phase of 20 hours and needed a longer time to adapt at 17°C and 12 °C with visible Lag Phases. Moreover this strain was not able to grow at 5°C.

To conclude *Agromyces sp*. could be considered as a mesophilic micro organism.

2.2 Yeasts

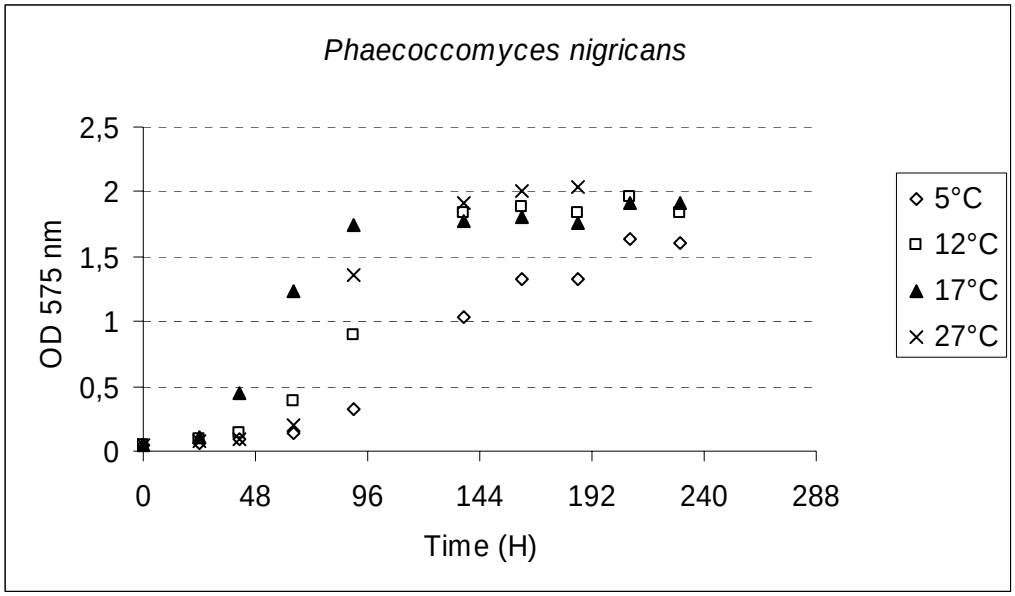


Figure 5.11: Growth of *Phaecoccomyces nigricans* at 5, 12, 17 and 27°C

Figure 5.11 presents the results obtained for *Phaecoccomyces nigricans*. It shows that the optimal growth temperature of this strain was 17°C, the growth at 27°C was close to the one at 17°C but the strain needed more time to adapt. It grew also well at 12°C with a longer Lag Phase. And finally *Phaecoccomyces nigricans* was able to grow at 5°C.

These results indicate that this strain could be considered as a psychrotroph yeast.

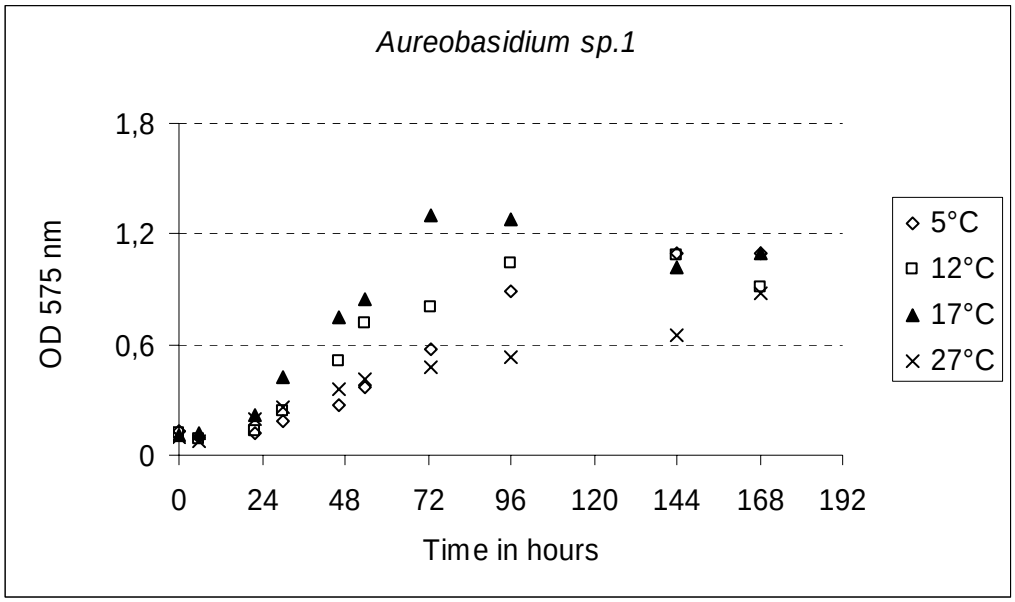


Figure 5.12: Growth at 5, 12, 17 and 27°C for *Aureobasidium sp 1*

Figure 5.12 presents the results obtained for *Aureobasidium sp.1*

The optimum growth temperature for this strain was 17°C. This yeast had a similar growth at 12°C and 5°C with lower OD values and presented a lower growth at 27°C.

These observations led to classify this strain as a psychrotroph yeast.

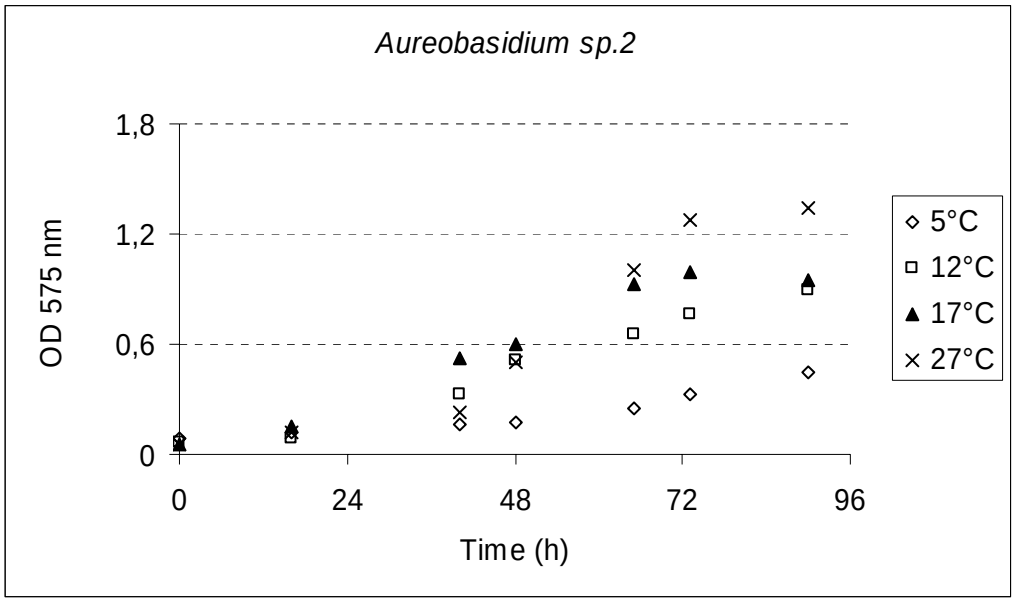


Figure 5.13: Growth of *Aureobasidium sp 2* at 5, 12, 17 and 27°C

Figure 5.13 presents the results obtained for *Aureobasidium sp2.* For this strain the growth at 17 and 27°C was similar with just higher OD for 27°C. But the strain seemed to start quicker at 17°C, indicating its optimum temperature should be 17°C.

The growth was also observed at 12°C with OD values close to the ones observed at 27 and 17°C. To conclude this strain was able to grow at 5°C

These observations led to classify this strain as a psychrotroph yeast.

3 Discussion and conclusion

According to Prescott et al. 2003, micro organisms could be classified depending on their growth with temperature.

Psychrophilic micro organisms can grow at 0°C and have an optimum growth temperature of 15°C or less.

Psychrotroph micro organisms (also called psychrotolerant) can grow between 0 and 7°C, have an optimum growth temperature between 20 and 30°C and a maximal one close to 35°C.

Bacteria with an optimal temperature between 20 and 45°C and a minimum growth temperature of 15°C are mesophilic micro-organisms.

Regarding these definitions it is difficult to classified exactly the arctic strains because some temperature missing.

In fact, we had laboratory constraint, no incubator was able to work at zero and it was possible to test only 4 temperatures in parallel, so the low temperature (5,12 and 17°C) were tested preferentially.

But the results obtained permits to underline some trends.

On 13 micro organisms, none of them had optimum temperature of 15°C or less, so none of them were strictly psychrophilic micro organisms.

Four bacteria (*Bacillus sp.*, *Brevundimonas sp.*, *Micromonospora sp.*, *Sandarakinorhabus limnophila 94,5%)* and the three yeasts had a psychrotroph behaviour with an observable growth at 5°C.

Two other bacteria (*Sandarakinorhabus limnophila 95,2%* and *Hydrogenophaga sp.*) gave results between a psychrotroph and mesophilic behaviour , the first one had a low growth at 7°C, and the second one was not able to grow at 5°C but was really disturbed at 27°C.

Finally, the four last strains (*Chelatococcus sp.*, *Moraxella sp.*, *Paenibacillus sp.* ,*Agromyces sp.*) had a mesophilic behaviour with no growth at 5°C and optimum growth temperature of 27°C.

To conclude, on the 13 micro organisms isolated from snow, 7 (4 bacteria and 3 yeasts) were able to grow at 5°C. This result indicates that these strains are probably able to grow during spring snowmelt in Arctic.

Once the physiology of the microorganisms characterized, their tolerance to mercury was investigate by performing Minimum Inhibition Concentration measurements.

The following chapter presented the MIC measurements obtained for both yeasts and bacteria.

Chapter 6: Mercury Minimum Inhibition Concentration for micr-organisms isolated from snow

1 Introduction of the chapter

After isolation of micro organisms from Arctic snow and investigation of their growth in chapter 5. We focused on the behaviour of micro organisms with mercury as this pollutant was known to be deposited onto snow each spring.

Minimal Inhibition Concentration (M.I.C.) corresponds to the minimum concentration of a pollutant which inhibits micro organisms growth. This kind of measurement permits to know until which level micro organisms tolerate mercury.

This measurments were performed on bacteria and yeasts with $HgCl_2$ as mercury source.

The results obtained are presented in the following parts, results for bacteria and yeasts are presented separately.

2 Arctic bacteria

Measurements were performed on 8 bacteria. MIC for *Brevundimonas sp* and *Sandarakinorhabdus limnophila 94,5%* could not been determined because of laboratory problems.

The results obtained are presented in table 6.1.

Strains	MIC ($mg.L^{-1}$ of $HgCl_2$)
Chelatococcus sp.	0,05
Agromyces sp.	0,1
Bacillus sp.	0,4
Micromonospora sp.	0,4
Hydrogenophaga sp.	0,4
Paenibacillus sp.	0,4
Moraxella sp.	1
Sandarakinorhabdus limnophila 95,2%	1

Table 6. 1 : Mercury Minimal Inhibition Concentration of $HgCl_2$ measured for arctic bacteria (from Marusczak 2007)

Three trends can be observed regarding these results,

- Strains with MIC inferior or equal to 0,1 $mg.L^{-1}$ of $HgCl_2$ (*Chelatococcus sp.* and *Agromyces sp.*)
- Strains with MIC at 0,4 $mg.L^{-1}$ of $HgCl_2$ (*Bacillus sp.*, *Micromonospora sp.* and *Hydrogenophaga sp.*)
- Strains with MIC at 1 $mg.L^{-1}$ of $HgCl_2$.(*Moraxella sp.* and *Sandarakinorhabdus limnophila 95,2%)*

For *Chelatococcus sp.* and *Agromyces sp.*, MIC of 0,050 $mg.L^{-1}$ and 0,1 $mg.L^{-1}$ were low and corresponded to 0.18 $\mu mol.L^{-1}$ 0.36 $\mu mol.L^{-1}$.

These results were ten times lower than the MIC value found for a strain already isolated from snow *Psychrobacter* which had a MIC of 2 $\mu mol.L^{-1}$ in the literature (Vetriani et al. 2005).

For Strains *Micromonospora sp.*, *Hydrogenophaga sp.* and *Paenibacillus sp*., MIC of 0,4 $mg.L^{-1}$, it corresponds to 1,47 $\mu mol.L^{-1}$ which was little lower than *Psychrobacter* MIC but in the same range.

The value for *Bacillus sp*. at 0,4 $mg.L^{-1}$ corresponds to 1,44 $\mu mol.L^{-1}$, which is lower than in the literature where reported MIC for *Bacillus sp*. was 5 $\mu mol.\ L^{-1}$ (Vetriani et al, 2005).

But the value observed by Vetriani et al (2005) was on a strain isolated from a deep sea hydrothermal vent so this strain was probably more exposed to mercury than our.

Regarding strains *Moraxella sp.* and S*andarakinorhabdus limnophila 95,2%*, MIC of 1 $mg.L^{-1}$ corresponds to 3.6 $\mu mol.L^{-1}$. As these strains were rare ones and their MIC for mercury have never been determined before and it's not possible to compare this value with those of the literature.

Regarding MIC results there were neither correlation between Gram – and Gram + nor depending on the phylogeny.

This work shows that polar strains had different behaviour toward mercury. Some of them had high MIC for strains supposed to live in remote environment and some others were really sensitive to mercury.

These differences raise the question of a possible adaptation of some bacteria to mercury deposition which occurs in their environment.

Lastly these mercury inhibition concentrations were higher than those detected in the snow pack.

These observations confirmed bacteria were able to support mercury deposition in their ecosystem.

Therefore molecular interactions between bacteria and bioavailable mercury deposited during the AMDE could take place.

3 Arctic Yeast

The results obtained for the three yeasts are presented in table 6.2.

Strain	M.I.C. ($mg.L^{-1}$ of $HgCl_2$)
Aureobasidium sp.1(J)	20
Aureobasidium sp. 2(G)	25
Phaecoccomyces nigricans (M)	10

Table 6. 2 : Mercury Minimal Inhibition Concentration of $HgCl_2$ measured for arctic yeast

The results obtained shows the two *Aureobasidium* species had MIC values of 20 $mg.L^{-1}$ of $HgCl_2$ for strain 1 and 25 $mg.L^{-1}$ of $HgCl_2$ for strain 2.

Phaecoccomyces nigricans had a lower MIC value at 10 $mg.L^{-1}$ of $HgCl_2$.

Some studies on mercury toxicity on common yeast like *Saccharomyces cerevisiae* exist. Yannai et al (1991) found that 0,750 $mg.L^{-1}$ of $HgCl_2$ inhibits the growth of the yeast in Nelson medium.

Yang et al (2003) measured the MIC90 (minimum inhibitory concentration that suppressed 90% of the cell growth) in yeast peptone media (YP) enriched with sugar and in Synthetic Complete media (SC) some MIC90 values respectively 348,2 $mg.L^{-1}$ and 0,088 $mg.L^{-1}$.

But in these studies the results clearly depended on the incubation media used because of their ability to complex or not mercury. We could postulate in another growth medium results could be different.

4 General conclusion on the MIC measurements

The results obtained for both bacteria and yeast shows differences between the two types of micro organisms. Indeed MIC values were higher for yeasts than for bacteria.

For bacteria a resistance mechanism toward mercury exists, it is called the *mer* system which is governed by an operon (operon *mer*). The resistance was based on the synthesis of several proteins which were able to uptake divalent mercury and to transmit it to a reductase which was able to reduce divalent mercury to elemental mercury(Barkay et al, 2003).

For yeast the resistance phenomenon is less clear. They were known to be able to detoxify some pollutant by a storage in the vacuole (Gueldry et al, 2003).

In any case our work did not permit to understand mechanisms involved but gave informations on the mercury tolerance of the Arctic micro organisms.

To complete this study, presence of mercury resistance genes was investigated in the Artic bacterial strains. The results obtained were presented in the chapter 7.

Moreover, as the exact mechanisms involved between yeast and mercury were not well known, an interaction experiment with mercury and methylmercury was performed on the yeasts and presented respectively in the chapter 8 and 9.

Chapter 7: Resistance genes in arctic bacteria

1 Introduction of the chapter

As detailed in the chapter 3 part 4. The resistance phenomenon in bacteria is based on synthesis of different proteins which will uptake divalent mercury and reduce it to its elemental volatile form.

To underline the presence or absence of *mer* genes in Arctic bacteria, we try to amplify a portion of the resistance genes with Polymerisation Chain Reaction (PCR).

For Gram negative bacteria (*Chelatoccocus sp., Brevundimonas sp. Hydrogenophaga sp., Moraxella sp., Sandarakinorhabus limnophila 94.5 and 95.2%)* a part of the merA and merR gene were targeted and amplified. *merA* codes for the mercuric reductase MerA which is able to reduce divalent mercury to elemental mercury, and merR codes for the repressor of the operon which is inhibited in presence of mercury (see chapter , part 4.3.1 for more details).

For Gram positive bacteria (*Bacillus sp*, *Micromonospora sp*., *Paenibacillus sp* and *Agromyces sp*.) it was a part of *merA* genes which was targeted.

When PCR products were obtained they were controlled by cloning in a plasmid and before being sequenced.

This genetical approach permitted to underline the presence of resistance genes in only two Gram negative bacteria *Chelatococcus sp* and *Moraxella sp*. results for the other strains were all negative so the following part presents the results obtained for these two positive bacteria.

2 Results

PCR products were detected in only two Artic strains : *Chelatococcus sp.* and *Moraxella sp.*

Figure 7.1 presents the electrophoresis gel obtained after migration of the pCR®II-TOPO® recombinant plasmid obtained with these two strains.

Before migration the recombinant plasmids were digested by EcoR1.

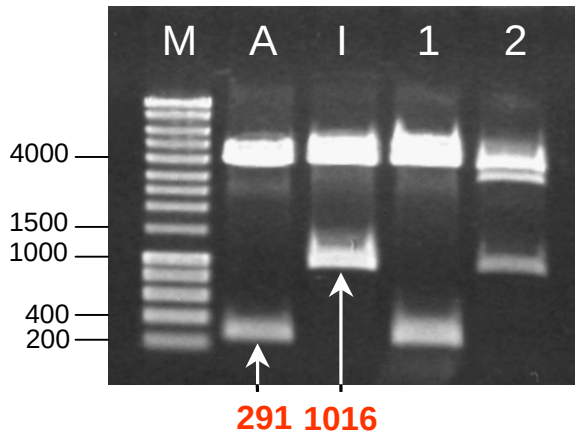


Figure 7. 1 : Restriction profiles of the pCR®II-TOPO® recombinant plasmid, after a digestion by EcoR1 and migration on agarose gel 0,8%. (Maruszcak, 2007)

On this figure:

- **M** corresponds to the Kb Ladder (Smart Ladder, Eurogentec ™)which indicates the size of the various fragments.
- **A** corresponds to the digestion of the plasmid carrying the insert of PCR product obtained from the genomic DNA of the strain *Chelatococcus sp.*.
- **I** corresponds to the digestion of the plasmid carrying the insert of a PCR product obtained from the genomic DNA of the strain *Moraxella sp.*.
- **1** corresponds to the positive control for the 291 bp fragment of *merA* genes from *Cupriavidus* metallidurans CH34
- **2** corresponds to the positive control of the 1016 bp fragment of *merR* gene from *Cupriavidus metallidurans CH34*

Figure 7.1 shows a 291 bp DNA fragment for strain *Chelatococcus sp* (A) which was the same that the one detected in control 1. This result indicated *Chelatococcus sp.* possessed *merA* genes as *Cupriavidus metallidurans* CH34 did.

Figure 7.1 also shows a 1016 pb DNA fragment for *Moraxella sp.*(I) which is the same as control 2. This result indicate that this strains has mer R genes as *Cupriavidus metallidurans* CH34.

3 Discussion and Conclusions

These results permitted to underline that *Chelatococcus sp* and *Moraxella sp.* possessed a part of the *mer* operon.

Therefore these strains could probably reduce inorganic mercury to gaseous elemental mercury.

In the environment, mercury resistance genes were known to be widely distributed in environmental bacterial strains (Misra 1992) and bacteria isolated from permafrost were already identified as *mer* operon carrier (Mindlin et al, 2005).

The fact the genes were not detected in the other bacteria did not necessary mean that they were not resistant. Indeed the *mer* gene sequence could have diverged from the primers tested.

We saw in chapter 6 that *Chelatococcus sp* had a MIC for $HgCl_2$ of 0,05 $mg.L^{-1}$ which was the lowest MIC values for arctic strains, it is surprising to see that these bacteria possess a part of the *mer* operon. The MIC result indicates that this strain not tolerate mercury so well, so maybe the part of the operon detected in its genome was not active.

Finding resistance genes in *Moraxella sp.* was less surprising because this strains had the higher MIC value (1 $mg.L^{-1}$)

In any case, strains isolated from snow could have a part of the *mer* operon, so these strains should be able to reduce divalent mercury to elemental gaseous mercury. As this phenomenon could occur in snow in springtime (Poulain et al. 2004), this result led to think that bacterial content of snow could have a role in the volatilisation of mercury from surface snow.

After the investigation about the toxicity of mercury on the micro organisms isolated from snow and the search of potential resistance to this metal in bacterial strains, and as this kind of research could not be performed on yeast because the mechanisms of interaction between yeast and metal are not well understood.

Research on the behaviour of yeast strains with low amount of mercury were performed, this work was presented in the following chapter.

Chapter 8: Behaviour of yeasts with inorganic mercury

1 Introduction of the chapter

As exposed before, behaviour of yeast with mercury is not well understood, the exact mechanisms between this metal and yeast cells is still unclear. In addition it was not possible to use molecular tools like for bacteria.

To investigate the interaction between environmental levels of mercury in the range of ng.L^{-1} and yeast, an experiment was performed using mercury isotopes. This experiment was presented in the chapter 3 part 5.2.

Briefly, each yeast was grown as pure culture in R2 liquid medium at 17°C and 200 rpm during 4 days. To start the experiment, inorganic ^{199}mercury was spiked in each culture to assess a final concentration of 100 ng.L^{-1}. Isotopic mercury was used in order to detect the formation of methylmercury during the experiment.

At different sampling times (0 hour, 3 hours, 24 hours, 48 hours and 96 hours) a 10 mL aliquot was sampled.

This aliquot was filtrated on 0,22 µm cellulose acetate filter to remove yeasts cells from the medium, the filtrate was called fraction 1 and corresponds to mercury remained in solution.

Then the filter was rinsed by 10 mL of EDTA 20 mM during at least 30 seconds to remove mercury easily exchangeable from the yeast cell wall. This EDTA rinse was called fraction 2. After this step all mercury bound to cell wall was supposed to be removed.

Finally the filter was extracted by 10 mL of 10% hydrochloric acid and 10 min sonication, this corresponded to fraction 3 which was supposed to give the mercury content retained by the cells.

To validate this protocol it was first applied on the growth medium alone without yeasts, and then on the three Arctic yeasts.

2 Behaviour of inorganic mercury in the Growth medium without yeasts

This part presents the results obtained with the R2 medium without yeast.

As no inorganic ^{199}mercury was detected in the fraction 2 the following results presents mercury concentrations only in fraction 1 and 3.

These results compared to the one obtained with yeasts permitted to show that mercury behaviour was not the same in a growth medium with and without yeasts

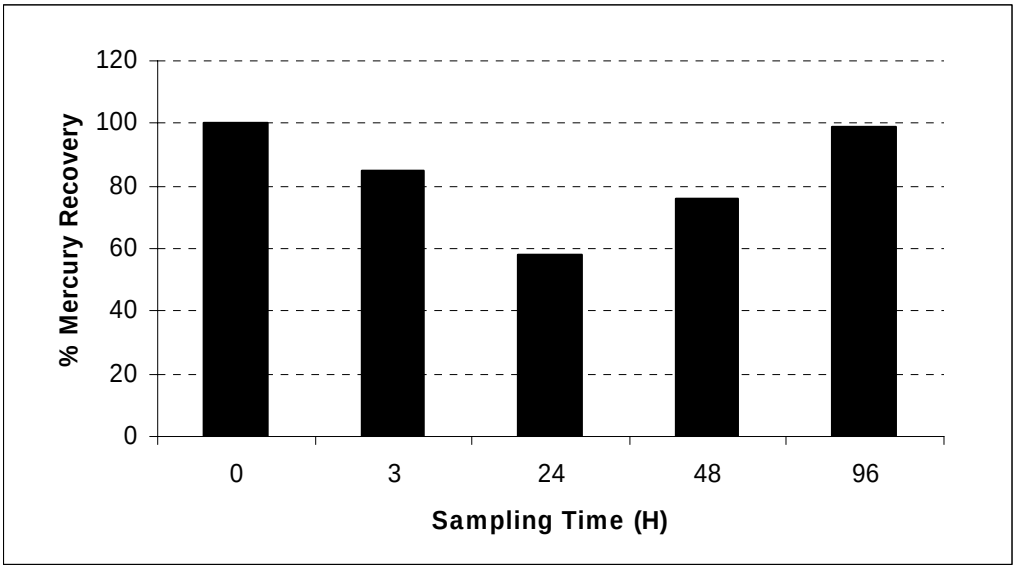


Figure 8. 1: Percentage of recovery of inorganic ^{199}mercury added, in the medium without yeast at different sampling times (fraction 1)

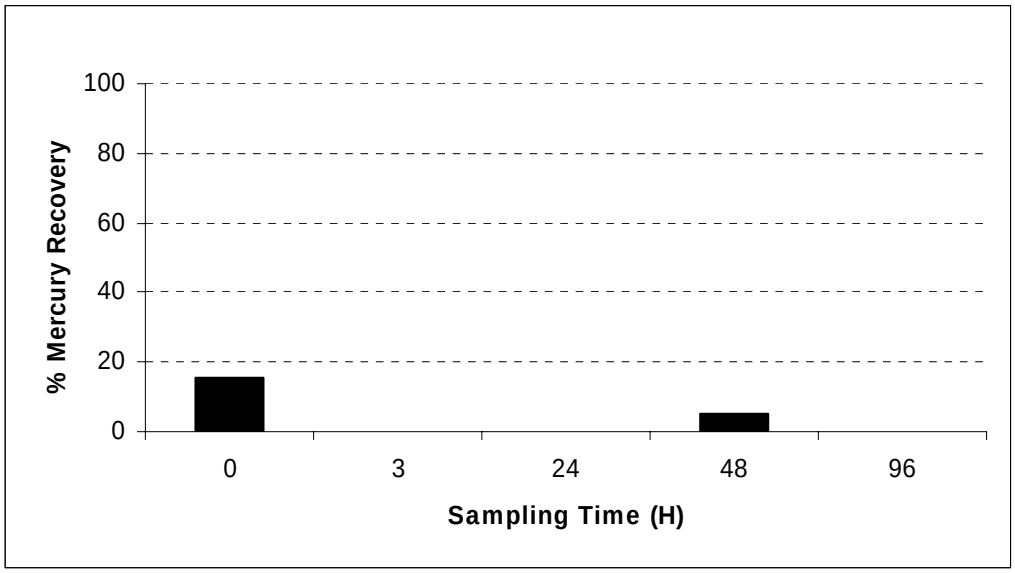


Figure 8. 2: Percentage of recovery of inorganic ^{199}mercury added, after extraction of the filter by 10% HCl and 10 min sonication at different sampling times. (fraction 3)

Figure 8.1 shows inorganic ^{199}mercury was detected at the different sampling times. The method used permitted to recover at least 58 % of the mercury introduced.

At 96 hours the amount of mercury detected was 100% of the amount added while it was less at 3, 24 and 48 hours, this could be either a loss of mercury during sampling for these three sampling times or a memory effect of the sampling system i.e. a part of mercury could remained on the sampling system in spite of the BrCl rinse.

There were several steps between sampling and mercury analysis. The sample was taken with a 10 mL plastic pipet tip, then transferred in a plastic syringe, and filtrated in a plastic filter holder and at the end collected in glass tubes. These steps could have led to little losses of mercury.

Moreover the samples were transported from the laboratory in France to the one in Italy, then melted and analysed. Even if a big care was taken all along the transport of the samples this way to work was maybe not the easiest one.

But finally these results indicated that inorganic ^{199}mercury was detectable all along the experiment in the medium.

Figure 8.2 indicates that once digested by 10% HCl and 10 min sonication the filter release 15% of the mercury added at sampling time zero and 5 % at 48 hours. This indicates that the filter could keep a little amount of mercury less than 15%.

As already said before, no inorganic ^{199}mercury was detected in the EDTA rinse. This indicates that no inorganic ^{199}mercury was bound on the cellulose acetate filter.

These first results with R2 medium indicate that the sampling method used permits to recover at least 58 % of the mercury added in the growth medium. The acetate cellulose filters used in this experiment retain a small part of mercury which could be released with EDTA rinse. No inorganic ^{199}mercury was released after extraction of the filter by 10% hydrochloric acid and 10 min sonication.

The results obtained for the fraction 1 underline a loss of mercury during sampling or a memory effect of the sampling system. If this second theory is the good one, it indicates a disappearance of mercury during the experiment.

This disappearance should be the result of chemical or photochemical reduction between component of the R2 medium and mercury; moreover the agitation of the medium could also be a problem for mercury stability.

In any case, the results obtained with the yeasts presented in the following parts gave so different profiles that some interpretation could take place.

3 Behaviour of yeast culture with inorganic mercury

This part presents the results obtained with the three Arctic yeasts: *Aureobasidium sp1*, *Phaecoccomyces nigricans*. and *Aureobasidium sp2*

3.1 With *Aureosbasidium sp 1.*

For this yeast, no inorganic ^{199}mercury was detected in the EDTA rinse indicating that mercury was not exchangeable on the cell wall (fraction 2).

So the following results present only mercury concentrations for the fraction 1 (mercury in the growth medium) and 3 (mercury inside the cells).

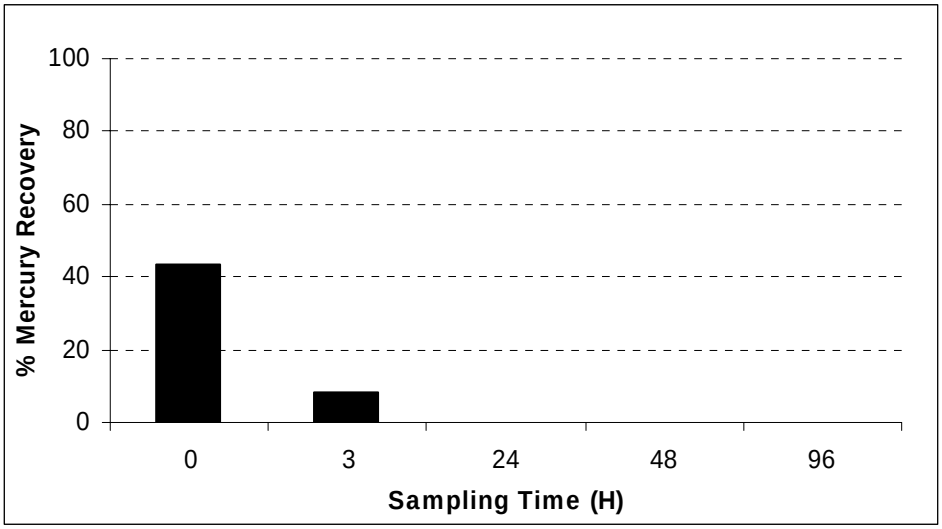


Figure 8. 3 : Percentage of recovery of inorganic ^{199}mercury added, in the medium of a *Aureobasidium sp1.* culture at different sampling times (fraction 1)

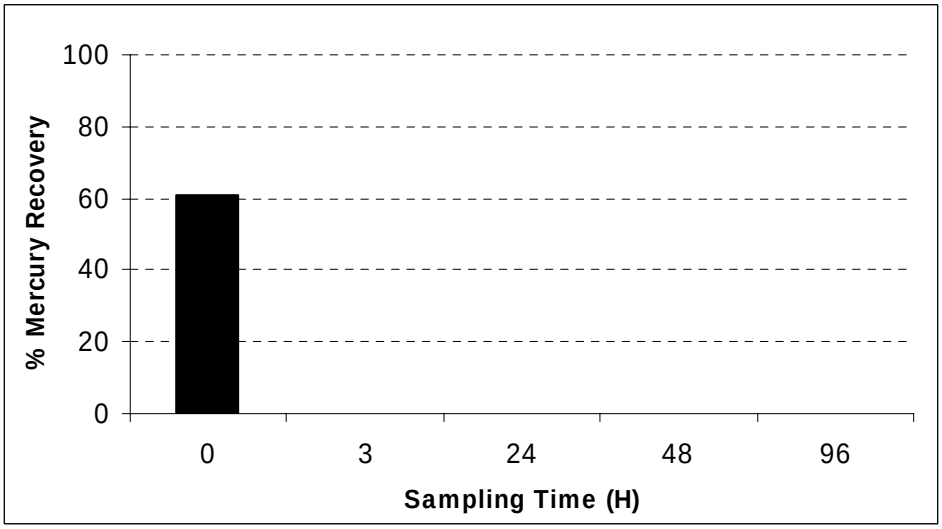


Figure 8. 4: Percentage of recovery of inorganic ^{199}mercury added after extraction of a filter containing *Aureobasidium sp1.* cells at different sampling times (fraction 3)

Figure 8.3 indicates that 43% of inorganic ^{199}mercury added in the culture was detected in the growth medium at the sampling time zero. At the sampling time 3 hours, 8% of inorganic^{199}mercury added was detected and no inorganic ^{199}mercury was detected for the other sampling times.

Figure 8.4 shows 60% of inorganic ^{199}mercury added was detected inside the cells at the sampling time zero but not during the rest of the experiment.

No methyl^{199}mercury or mercury complexes were detected in any fraction for this yeast.

In this experiment the partition of mercury between growth medium and yeast cells was clearly observable. At time zero, which means just after mercury addition, 40% of mercury was detected in growth medium and 60 % inside the cells (or associated with the cells). Three hours later, only 8% of inorganic mercury was detected in the medium only. So 92% of inorganic mercury added disappear from the system in 3 hours because of the presence of yeasts cells. No inorganic ^{199}mercury was released from the cell wall after the EDTA rinse.

In this experiment no methyl^{199}mercury or mercury complexes were detected.

One of the resistance phenomenon toward inorganic mercury observed for example in bacteria is based on the reduction of inorganic mercury to its elemental form which volatilize to the atmosphere (Barkay et al, 2003).

Considering this, the disappearance of mercury from the medium observed in this experiment and the fact that mercury was not detected link to the cells or inside it, leads to think *Aureobasidium sp1* cells were able to reduce divalent mercury in elemental gaseous mercury.

3.2 With *Phaecoccomyces nigricans*

For this yeast inorganic ^{199}mercury was detected in the 3 fractions. The following results present inorganic ^{199}mercury recovered in all of them.

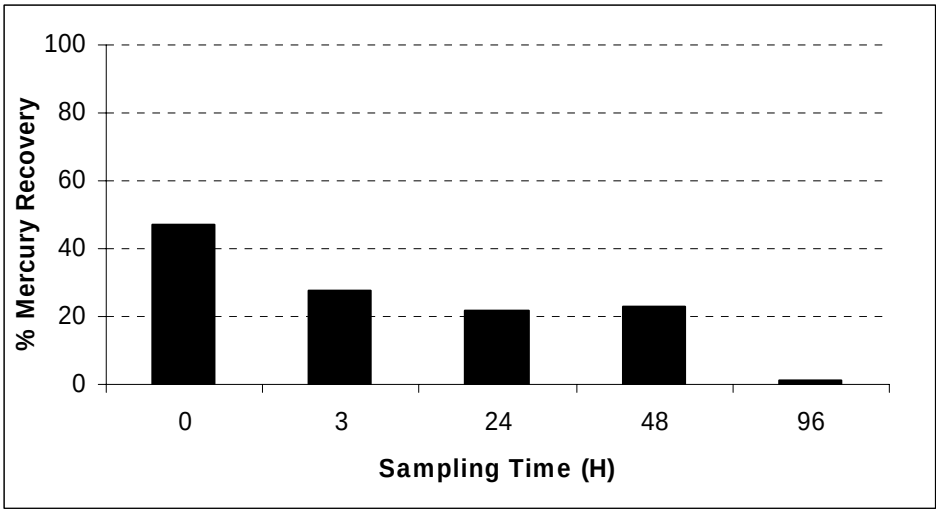


Figure 8. 5: Percentage of recovery of inorganic ^{199}mercury added in the medium of a *Phaecoccomyces nigricans* culture at different sampling times (fraction 1)

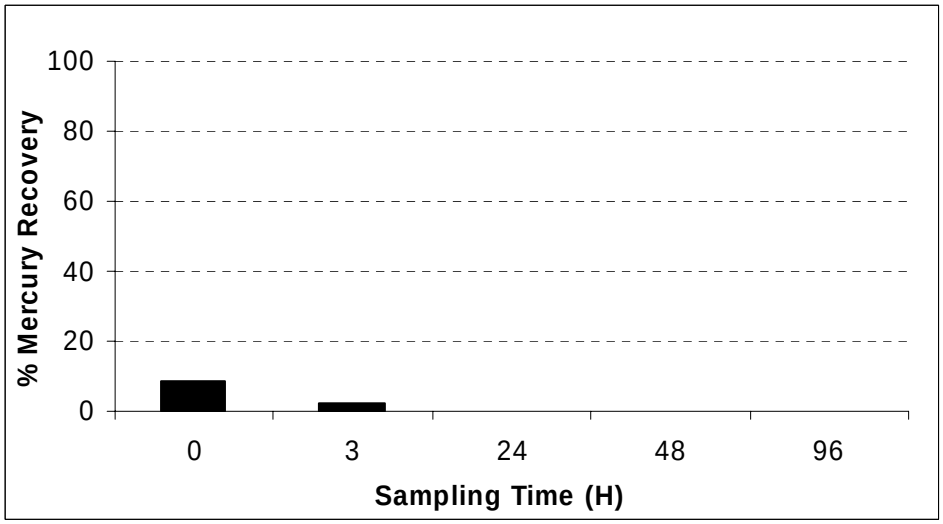


Figure 8. 6: Percentage of recovery of inorganic ^{199}mercury added in the EDTA rinse at different sampling times for a culture of *Phaecoccomyces nigricans* (fraction 2)

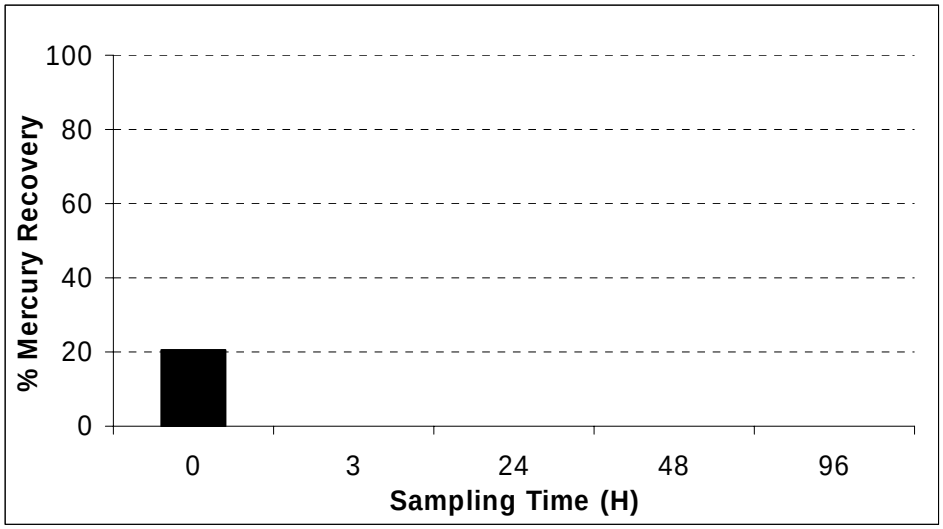


Figure 8. 7: Percentage of recovery of inorganic ^{199}mercury added after extraction of a filter containing *Phaecoccomyces nigricans* cells at different times (fraction 3)

Figure 8.5 shows inorganic ^{199}mercury added was detected in the growth medium of *Phaecoccomyces nigricans* all along the experiment.

Levels of mercury detected represented about 47% of the mercury added at the sampling time zero, then the equivalent of 27 % at the sampling time 3 hours; 21% at sampling times 24 and 48 hours.

Only 1% of inorganic ^{199}mercury added was detected at the sampling time 96 hours.

Figure 8.6 shows a small amount of inorganic ^{199}mercury was detected in the EDTA rinse (fraction 2) at sampling times at 0 and 3 hours respectively 8 and 1 % of the mercury added. No ^{199}mercury was detected for the other sampling times.

Figure 8.7 shows 20% of inorganic ^{199}mercury added was detected in the fraction 3 i.e. inside yeasts cells at the sampling time zero. No inorganic^{199}mercury was detected at the other sampling times.

No methyl^{199}mercury or mercury complexes were detected in any fraction for this yeast.

Here the partition of mercury between growth medium, yeast cell wall and yeast cells was observable, at time zero 75% of the total amount of mercury was recovered (47 % in the medium, 8 % on the cell wall and 20 % inside the cells). At sampling time 3 hours, only 28% of mercury added remained in the system. In 3 hours a loss of 47% of the mercury added occurs.

A small part of mercury was retained by the cell wall and released by EDTA rinse.

This last observation combined to the detection of mercury in the fraction 3 shows a probable entry of the metal inside yeast cells. The disappearance of mercury from the system during the experiment as observe for *Aureobasidium sp1* indicates a probable reduction of the metal in its elemental gaseous form.

But in this case as mercury was detected in the growth medium at 24 and 48 hours the phenomenon seemed less effective than for *Aureobasidium sp.1*,in a *Phaecoccomyces nigricans* culture 96 hours were needed to see a total disappearance of mercury from the sytem.

3.3 With Aureobasidium sp 2

For this yeast as for *Aureobasidium sp1*, no inorganic ^{199}mercury was detected in the EDTA rinse indicating that mercury was not exchangeable with the cell wall.

Moreover no inorganic ^{199}mercury was detected inside the cells (fraction 3).

The following results present only mercury concentrations in the growth medium (fraction 1).

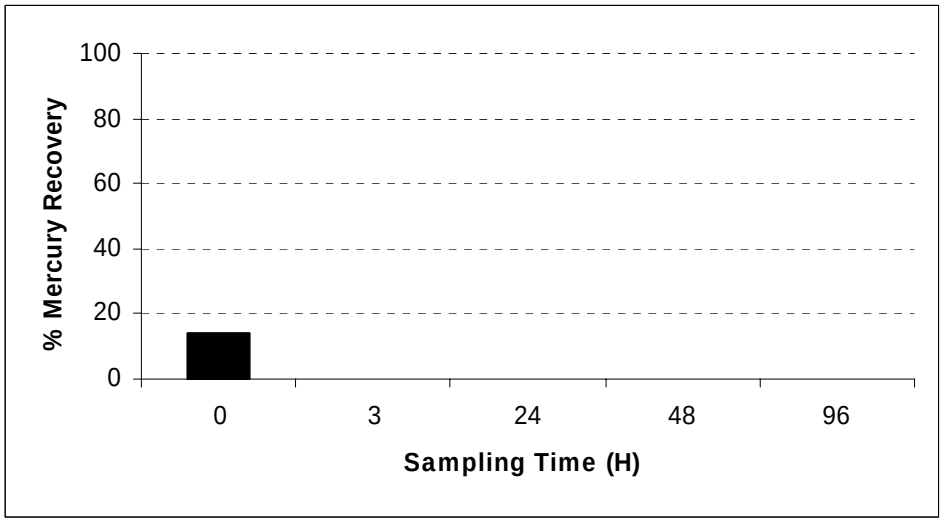


Figure 8. 8: Percentage of recovery of inorganic ^{199}mercury added in the medium of a *Aureobasidium sp2.* culture at different sampling times (fraction 1)

Figure 8.8 indicates that 15 % of inorganic ^{199}mercury added was detected in the growth medium at the sampling time zero, for the other sampling times no inorganic ^{199}mercury was detected.

No methyl^{199}mercury or mercury complexes were detected in any fraction for this yeast.

In this case a loss of mercury from the system occurs.

Sampling at time zero was performed few minutes after mercury addition, in this meantime 85% of the amount of mercury added disappears. The experiment for this yeast was performed in parallel with the other yeasts was no such disappearance was observed. Finally inorganic mercury was not detected absorb to the cell wall neither inside the cells.

As observed with the two other strains, these observations led to think to a reduction of inorganic mercury to elemental one but in this case, the phenomenon seems to take place in a very short time in the range of minutes.

Aureobasidium sp2 maybe have a resistance system which is activate very quickly as in mercury resistant bacteria where this kind of mercury reduction could be observed in few minutes (Serre et al,. 2004).

4 Discussion and conclusions about yeast behaviour with inorganic mercury

Interaction between yeast and metal ions was already studied in *Saccharomyces cerevisae* but with different metal ions such as Mn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+} or Zn^{2+} and with concentrations in the range of $mg.L^{-1}$. With these metals yeast cells seems to be able to adsorbs metal ions in 5 minutes because of their cell wall properties which gave complexation sites to the metal (Avery et al 1993; Madrid et al. 1995; Kambe-Honjoh et al. 1997). For other metal like cadmium a transport across the membrane seemed to occur but the exact mechanisms stayed unclear (Malik 2004).

Phenomenon involved in metal resistance in yeasts also stayed unclear, one of the ideas was the metal was transported and stored in the vacuole which decreases its toxicity (Gueldry et al. 2003).

Conversely very few studies existed on interaction between yeast and inorganic mercury.

The results obtained on the three yeasts shows similar trends, a disappearance of the amount of inorganic ^{199}mercury measured in the medium with the time, in opposition of what was observed in the growth medium alone.

No inorganic ^{199}mercury was detected on the cell wall for the two *Aureobasidium* species and a small amount detected for *Phaecoccomyces nigricans*.

And finally, inorganic ^{199}mercury was detected after an extraction of the filter containing yeasts cells for two of the three yeasts species only at the beginning of the experiment indicating a probable entry of the metal inside the cells.

These results suggest that as for thought for cadmium a cellular uptake of the metal probably occur. Moreover as observed in bacteria a reduction of inorganic mercury to elemental gaseous mercury seemed to take place in Arctic yeasts.

No methyl^{199}mercury or mercury complexes was detected in any fraction for any yeast during this experiment, indicating these strains were not able to methylate mercury or forme complex with it.

In perspective of this work, some complementary experiments are necessary to better understand and characterize the mechanisms involved in the interaction between yeast and mercury.

First the behaviour of mercury with R2 medium should be more studied to better control the loss of mercury.

Then a separation of the different cellular compartments of yeasts like vacuole or membrane and mercury measurements in there could gave idea of the exact fate of mercury inside the cell.

Finally we postulate that yeast cell wall had the same affinity for mercury that bacterial cell wall. Fein et al. 2001 manage to estimate the stability of complex formed by bacteria and metals ions, this kind of experiment should be performed on yeast cell wall to validate totally the protocol used.

Regarding the probable mercury reduction in gaseous elemental mercury performed by yeast strains, it is interesting to know that during spring in Arctic a big part of the divalent mercury deposited onto snow is rapidly reduced in elemental gaseous mercury.

This kind of phenomenon is supposed to be a chemical one, but regarding the results obtained yeast from snow could play a role in the reduction of mercury to elemental gaseous one.

To continue this work about yeasts and mercury, the interaction experiment was performed between methylmercury and yeasts to test if the strains were able to demethylate methylmercury the results are presented in the following chapter.

Chapter 9: Behaviour of yeasts with methylmercury

1 Introduction of the chapter

To continue the work about interaction between mercury and yeasts an interaction experiment with methyl^{201}mercury was also performed to test if Arctic yeast were able to demethylate mercury.

The experiment was the same as presented in chapter 8 but with methyl^{201}mercury.

Methylmercury concentrations in snow are very low down to 1 ng.L^{-1}, as it was not possible to work on such small amount, the methylmercury concentration in this interaction experiment was 150 ng.L^{-1}.

As for inorganic ^{199}mercury the experiment was first performed on the growth medium alone and then on pure culture of yeasts.

The experiment includes sampling at different times, filtration of the sample to assess methylmercury in the growth medium (fraction 1), rinse of the filter with EDTA to assess methylmercury easily exchangeable from the yeast cell wall (fraction 2) and extraction of the filter to assess methylmercury content inside the cells (fraction 3).

2 Behaviour of methylmercury in the Growth medium without yeasts

This part presents the results obtained with the R2 medium without yeast.

As no methyl^{201}mercury was detected in the fraction 3 the following results, obtained for the growth medium alone, presents mercury concentrations only in fraction 1 and 2.

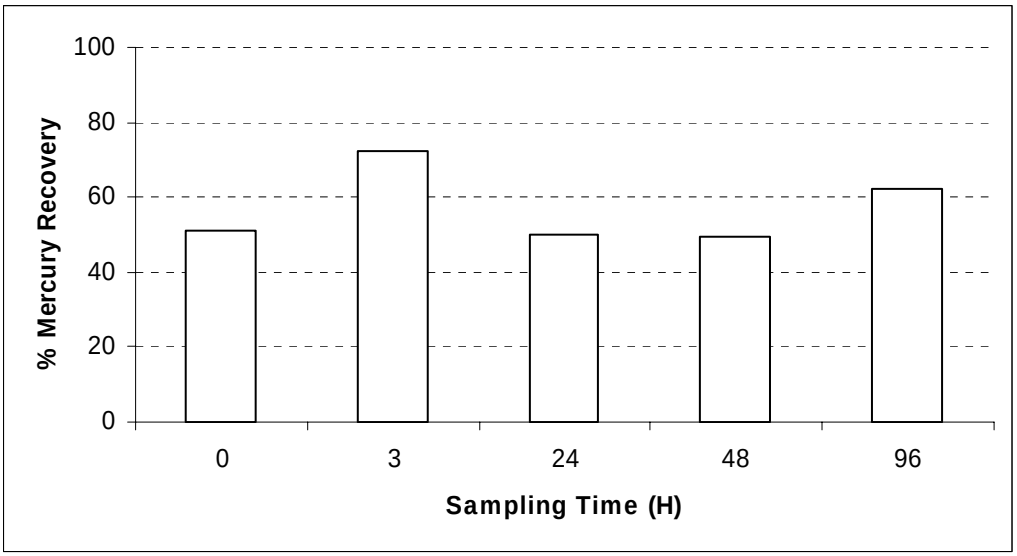


Figure 9. 1: Percentage of recovery of methyl^{201}mercury added with our sampling method in the medium without yeast at different sampling times (fraction 1)

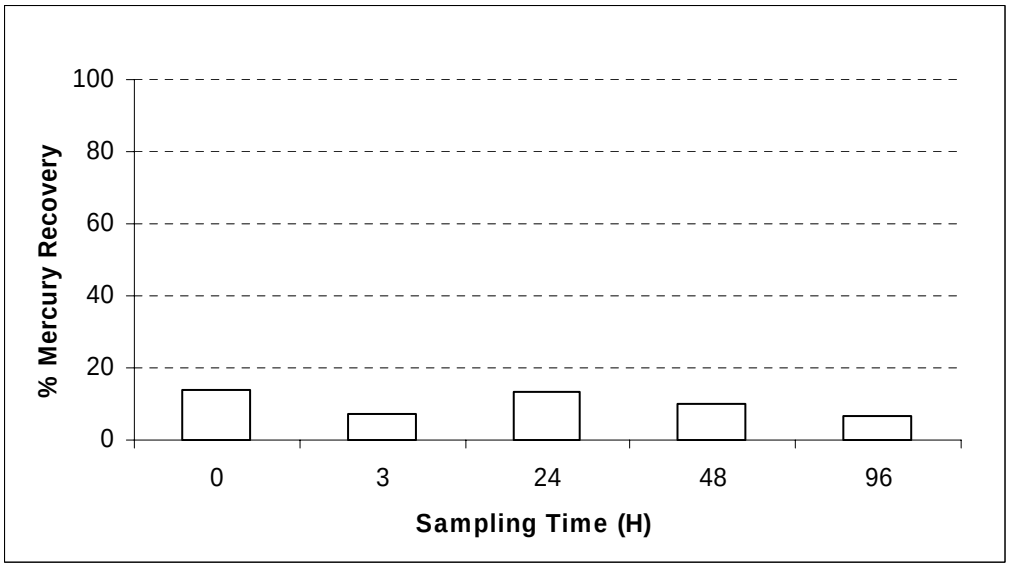


Figure 9. 2: Percentage of recovery of methyl^{201}mercury added with our sampling method in the EDTA rinse at different sampling times (fraction 2)

Figure 9.1 shows methyl^{201}mercury was detected at the different sampling times. The method permitted to recover at least 50 % of the methylmercury added at sampling times 0, 24 and 48 hours. At sampling times 3 hours 70% of methyl^{201}mercury added was detected and 60 % at sampling time 96 hours.

Figure 9.2 shows methyl^{201}mercury was detected once the filter was rinsed by EDTA 20 mM. Methyl^{201}concentrations represented 15% of the methyl^{201}mercury added at sampling time zero and 24 hours, 10% at sampling times 3 hours and 96 hours and 12 % at sampling time 48 hour. This indicates that cellulose acetate filter used in this experiment retained a small amount of methyl^{201}mercury released by the EDTA rinse.

As introduced before no methyl^{201}inorganic mercury was detected after the digestion of the filter by 10 % HCl and 10 min sonication.

By summing up the recovery percentages in the two fractions, a global recovery percentage indicates that at least 62 % of the mercury added was recover by the method used.

No inorganic ^{199}mercury was detected in any fraction.

The loss of methylmercury observed in these samples could be due to a chemical or photochemical demethylation in R2 medium, and then a reduction of the divalent mercury as observed in the previous experiment with inorganic mercury.

3 Behaviour of methylmercury in a culture of yeast

This part presents the results obtained with the three Arctic yeasts : *Aureobasidium sp1*, *Aureobasidium sp2* and *Phaecoccomyces nigricans*.

3.1 With *Aureobasidium sp1*

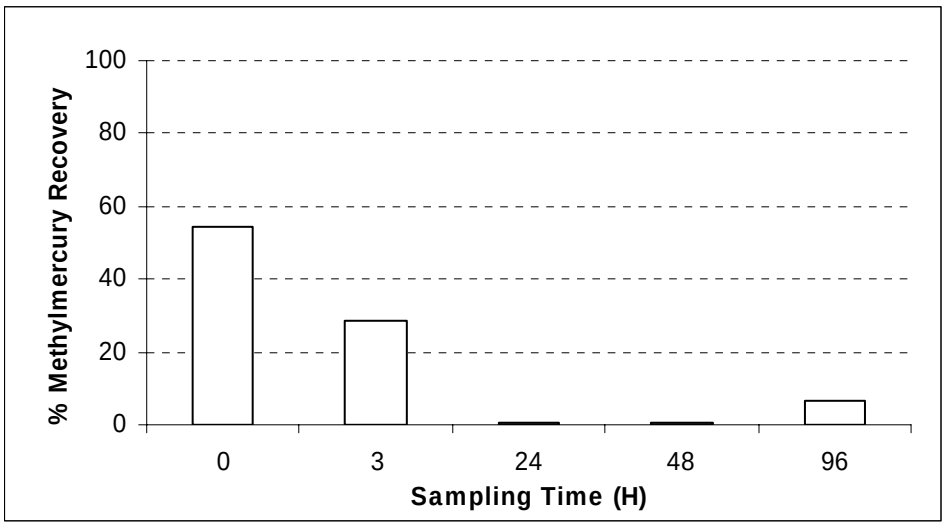


Figure 9. 3: Percentage of recovery of methyl^{201}mercury added in the medium of a *Aureobasidium sp1.* culture at different sampling times (fraction 1)

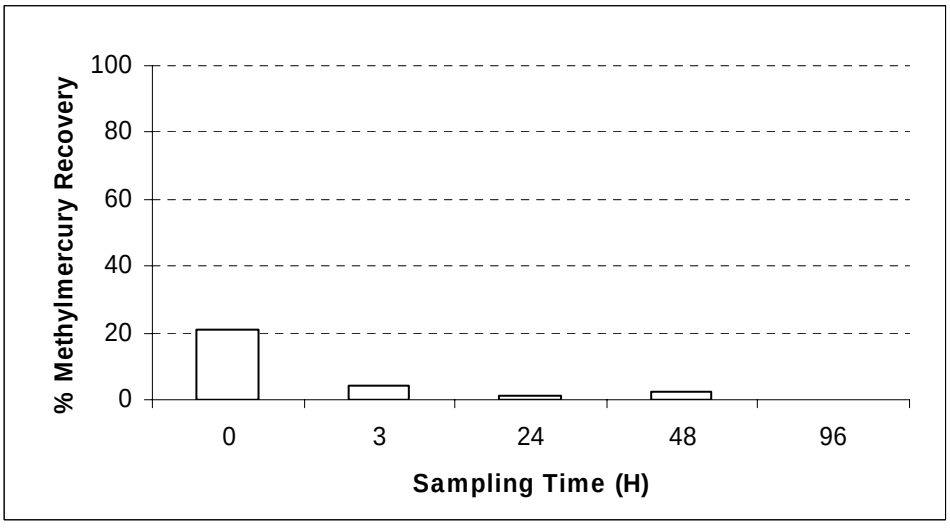


Figure 9. 4: Percentage of recovery of methyl^{201}mercury added in the EDTA rinse at different sampling times for a culture of *Aureobasidium sp1* (fraction 2)

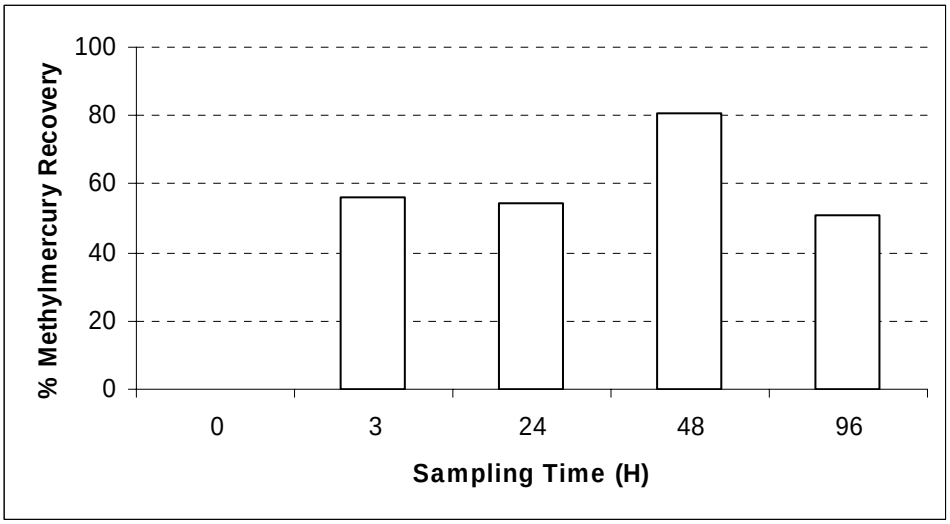


Figure 9. 5: Percentage of recovery of methyl^{201}mercury added after extraction of a filter containing *Aureobasidium sp1* cells at different times (fraction 3)

Figure 9.3 shows 52% of methyl^{201}mercury added was detected at the sampling time zero. At the sampling time 3 hours the amount of mercury detected was 30%, for sampling times 24 and 48 hours the amount detected was zero. For sampling times 96 hours a mercury amount corresponding to 10 % of the mercury added was detected.

This last data indicates that the values obtained at 24 and 48 hours probably underestimate the amount of mercury remained in the media which should be in the range of 10% at the end of the experiment.

Figure 9.4 shows 20% of methyl^{201}mercury added was detected in the EDTA rinse at the sampling time zero. For the other sampling times we detect low amount of mercury between 0 and 4%.

These results suggested that a part of the mercury was located on the cell wall at the beginning of the experiment.

Figure 9.5 shows that more than 50 % of methyl^{201}mercury added was detected inside the cell since the sampling time 3 hours and for all the other sampling times.

The results obtained with *Aureosbasidium sp1* gave clearly different profiles than the ones obtained for the growth medium alone.

They indicate that methyl^{201}mercury disappeared quickly from the growth medium in 24 hours, entered rapidly in the yeast cell and stayed stored inside the yeast without being demethylated.

3.2 With *Aureobasidium sp2*

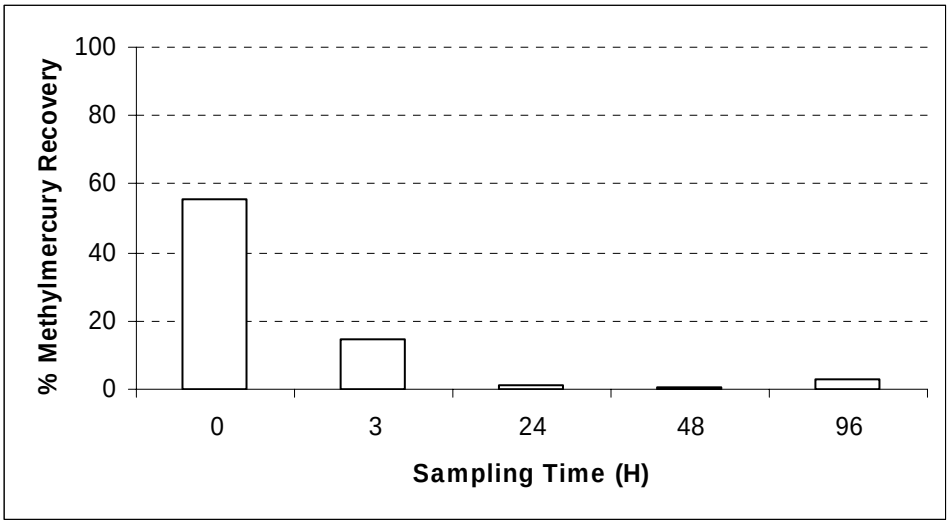


Figure 9. 6: Percentage of recovery of methyl^{201}mercury added, in the medium of a *Aureobasidium sp2.* culture at different sampling times (fraction 1)

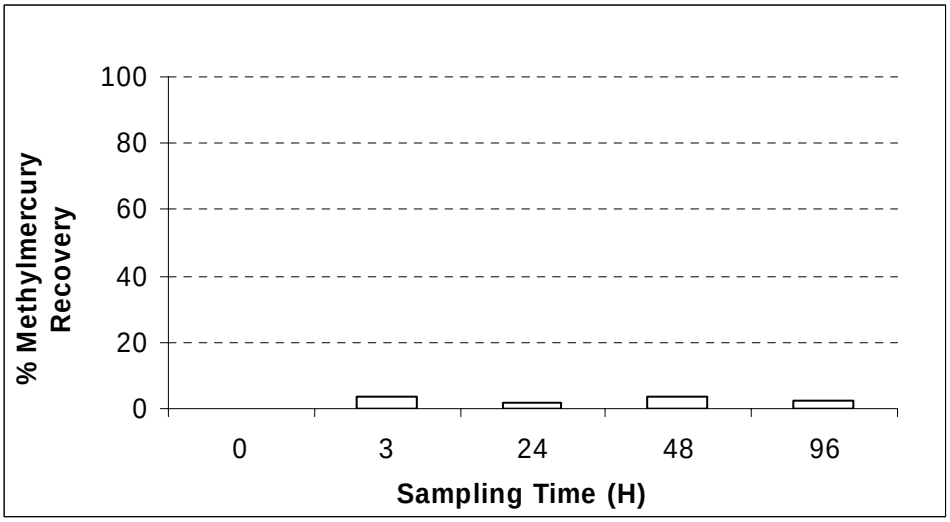


Figure 9. 7: Percentage of recovery of methyl^{201}mercury added in the EDTA rinse at different sampling times for a culture of *Aureobasidium sp2* (fraction 2)

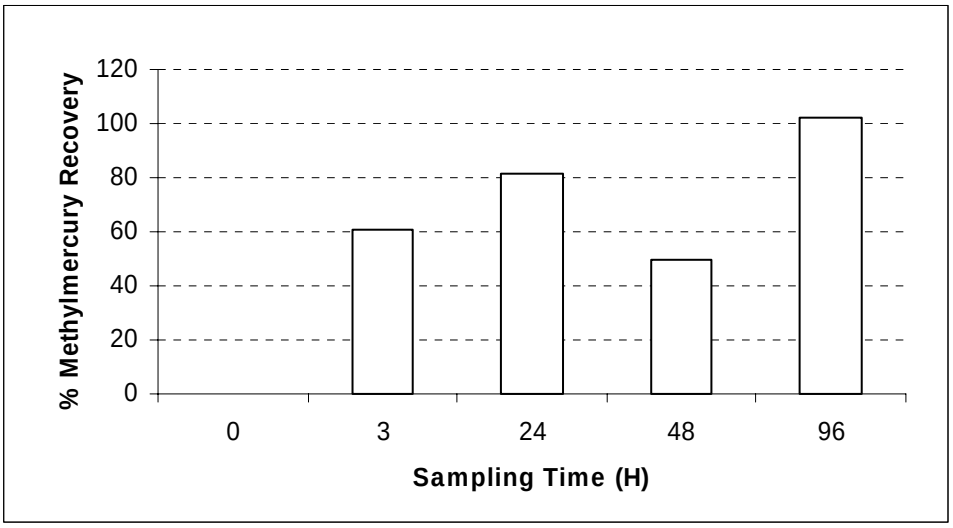


Figure 9. 8: Percentage of recovery of methyl^{201}mercury added after digestion of a filter containing *Aureobasidium sp2* cells at different times (fraction 3)

Figure 9.6 shows 58% of methyl^{201}mercury added was detected at the sampling time zero.

At the sampling time 3 hours the amount of mercury detected was 14%, for sampling times 24, 48 and 96 hours the amount detected was close to zero.

Figure 9.7 shows a small amount of methyl^{201}mercury added (less than 3%) was detected in the EDTA. This result suggested that a part of the mercury remained on the cell wall.

Figure 9.8 presents methyl^{201}mercury added detected inside the cells for *Aureobasidium sp 2.*, 60% were detected at sampling time 3 hours, 80 % at sampling time 24 hours, 50% at sampling time 48 hours and 100% at sampling time 96 hours.

The results obtained with *Aureobasidium sp2* shows the same trend as for *Aureobasidium sp1*.

Methyl^{201}mercury disappeared from the medium, entered the cell and stay stored inside. For this strain methyl^{201}mercury was detected on the cell wall indicated a probable transport across the membrane.

3.3 With *Phaecoccomyces nigricans*

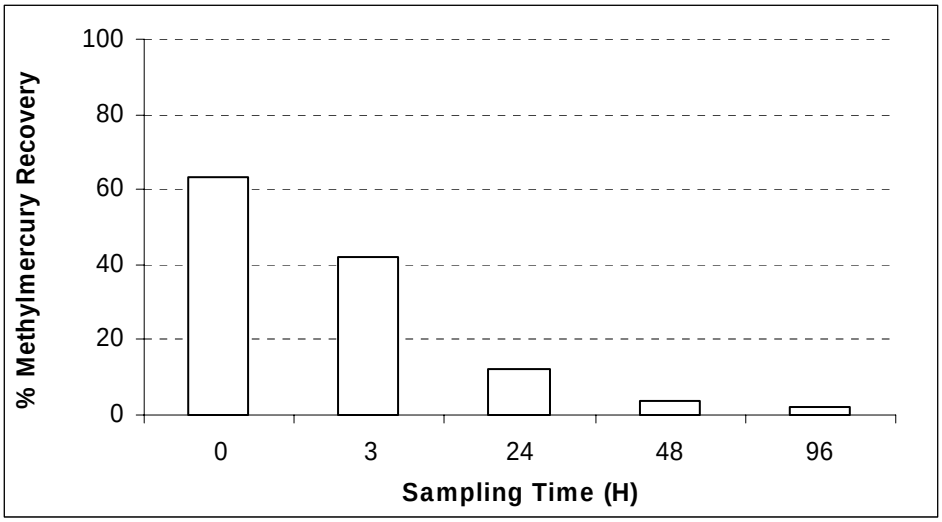


Figure 9. 9: Percentage of recovery of methyl^{201}mercury added, in the medium of a *Phaecoccomyces nigricans.* culture at different sampling times (fraction 1)

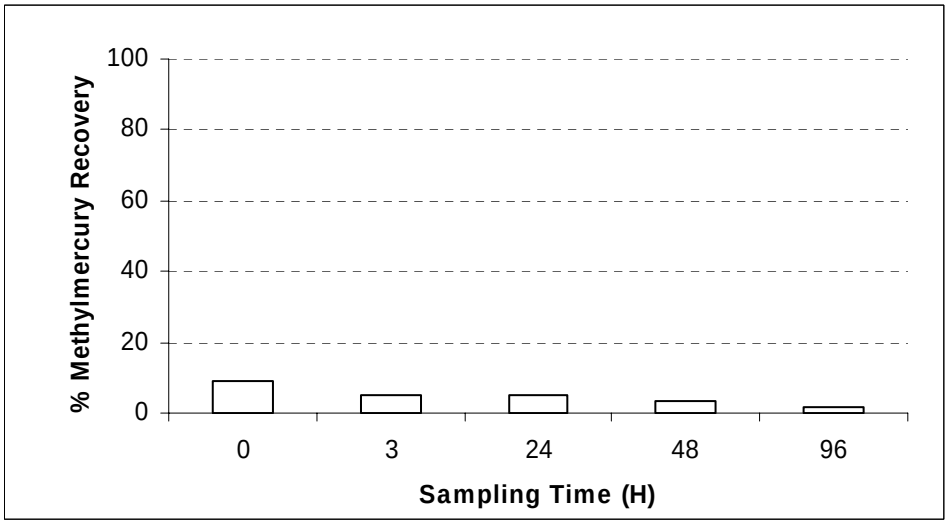


Figure 9. 10: Percentage of recovery of methyl^{201}mercury added in the EDTA rinse at different sampling times for a culture of *Phaecoccomyces nigricans* (fraction 2)

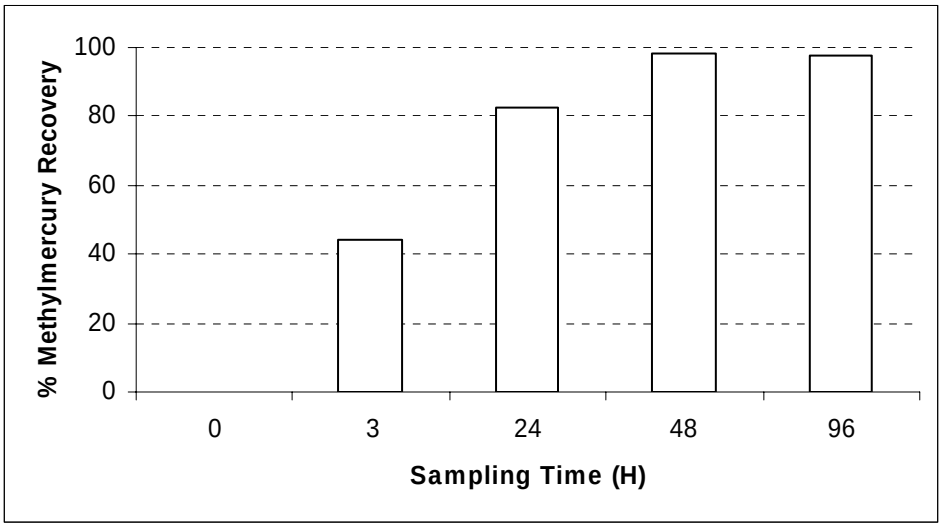


Figure 9. 11: Percentage of recovery of methyl^{201}mercury added after digestion of a filter containing *Phaecoccomyces nigricans* cells at different times (fraction 3)

Figure 9.9 shows 60% of methyl^{201}mercury added were detected at the sampling time zero At the sampling time 3 hours the amount of mercury detected was 40%, at the sampling time 24 hours 15% of the initial mercury concentration were detected.

The methyl^{201}mercury levels for sampling times 48 and 96 hours were inferior to 5%.

Figure 9.10 shows that 9% of methyl^{201}mercury added was detected in the EDTA rinse at the sampling time zero. The amount detected for the others sampling times represents less than 5 % of the total mercury added and seemed to decrease with the increase of time.

Figure 9.11 presents percentage of methyl^{201}mercury added detected inside the cells for *Phaecoccomyces nigricans*. as for *Aureobasidium* species methyl^{201}mercury was detected inside the cell since the sampling time 3 hours (40 % of methyl^{201}mercury added) and for all the other sampling times.

Results observed for *Phaecoccomyces nigricans* were comparable the ones obtained for *Aureobasidium* species.

For this strain, methyl^{201}mercury was detected on the cell wall.

The concentrations detected started at 10 % and decrease with the time. These observations combined to the results obtained on Figure 9.11 indicated an internalisation of methyl^{201}mercury by yeasts cells.

4 Discussion and conclusions about yeasts behaviour with methylmercury

The sequestration of methylmercury by yeasts cell was already observed by Madrid et al, 1995 with *Saccharomyces cerevisiae*. In this study yeast cells were able to demethylate methylmercury but the amount of methylmercury tested was in the range of mg.L^{-1} as this kind of mercury level was close toxic concentration (see chapter 6) the demethylation could be a way to detoxify methylmercury.

The results obtained in our experiment with low level of methylmercury (ng.L^{-1}) showed the same trend for the three yeasts: a rapid disappearance of methyl^{201}mercury from the growth medium in 24 hours and in the meantime appearance of methylmercury inside the cells where it stayed store in without being demethylated.

For *Aureobasidium* species no methyl^{201}mercury was detected on the cell wall which was the case for *Phaecoccomyces nigricans*. For this strain until 10 % of the methyl^{201}mercury could be detected on the cell wall.

These results underline an entry of mercury in the cell.

This experiment permits to have an idea of the behaviour of Arctic yeast with environmental amount of methylmercury.

But as the experiment interaction with inorganic mercury presented in chapter 8, some complementary experiment are necessary to identify the exact mechanisms involved between yeast and methylmercury.

In this case too, the behaviour of methylmercury with R2 medium should be more studied to better control the loss of methylmercury in the medium without yeast.

As for inorganic mercury, the stability of complex formed by yeast cells and methylmercury should be evaluated like Fein et al. 2001 did with *Bacillus subtilis*.

Some complementary experiments with a separation of the different cellular compartments of yeasts like vacuole or membrane and mercury measurements in there could gave idea of the fate of methylmercury inside the cells.

To conclude, these results suggest that once formed methylmercury in the environment is quickly absorbed by micro organisms.

After the work on micro organisms isolated from Ny-Ålesund snow and interaction with mercury presented in previous chapters 4, 5, 6, 7, 8 and 9.

The work on micro organisms in Artic was followed by analysing the total mercury content in planktonic samples collected in Ny-Ålesund's fjord : the Kongsförden during the field campaign in spring 2007

The results are presented in the chapter 10.

Chapter 10: Total mercury measurements in planktonic samples during a field campaign

1 Introduction of the results

Because there's few data about mercury in plankton in Svalbard, the aim of this work was to measure total mercury in planktonic sample during the field campaign in spring 2007.

During this campaign gaseous elemental mercury in the atmosphere was monitored such as divalent mercury in the snow.

In parallel to these measurements plankton was sampled every week and separated in 5 class depending on the size (<30µm; 30-63 µm; 63-200 µm; 200-500 µm and > 500µm).

The sampling dates were 25th of April, the 3rd of May, the 10th of May, the 16th of May, 23rd of May, the 30th of May, the 6th of June and the 13th of June.

Total mercury was measured in each size class in order to see if the mercury deposited during the AMDE had an impact on the total mercury content in plankton. On these samples methylmercury measurements were impossible because under the detection limit.

The results are presented in the following chapter, data concerning mercury in the air and in the snow are presented first. After the evolution of total mercury concentration in the different size fraction of plankton depending on the sampling date are presented.

Finally the mean by size fraction was calculated to see if mercury content was different depending on size.

The results for mercury in plankton are in ng/g of dry weight, also written ng.g of d. w.

2 Gaseous elemental mercury monitoring in Ny-Ålesund atmosphere during spring 2007.

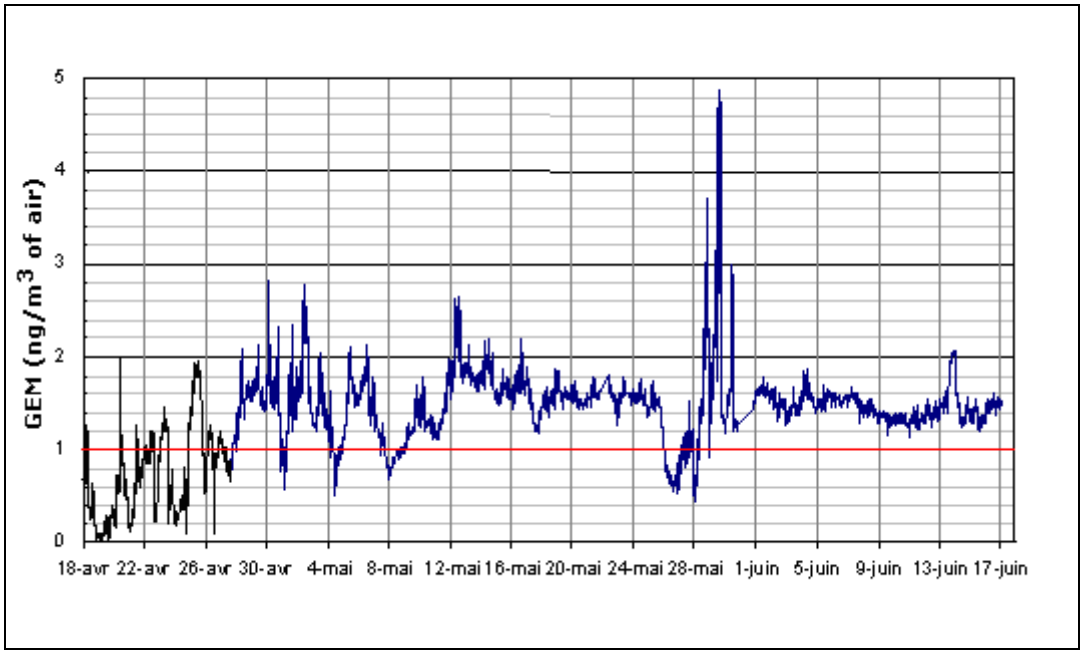


Figure 10.1 : Gaseous Elemental Mercury monitored in Ny-Ålesund Atmosphere in spring 2007. Black curve represents measurements performed by NILU (Norwegian Institute for Air Research, Norway) and blue curve represents measurements performed by the CHIMERPOL Team. The red line represents the 1 ng/m^3 level under which Atmospheric Mercury Depletion Event occurs.

When gaseous elemental mercury concentration in the atmosphere was under 1 ng.m^3 of air it means that an Atmospheric Mercury Depletion Event occurred.

Figure 10.1 shows gaseous elemental mercury concentrations varied depending on time. On the 18th of April the concentrations were very low close to zero, this period between 18th and 26th of April corresponded to an Atmospheric Mercury Depletion Event. After this period concentrations stayed stable around 1,7 ng.m^3 of air and some depletion events less important occurred on the 1st , the 5th, the 8th of May, and between the 26th and the 28th of May.

3 Total mercury in Ny-Ålesund snow during spring 2007.

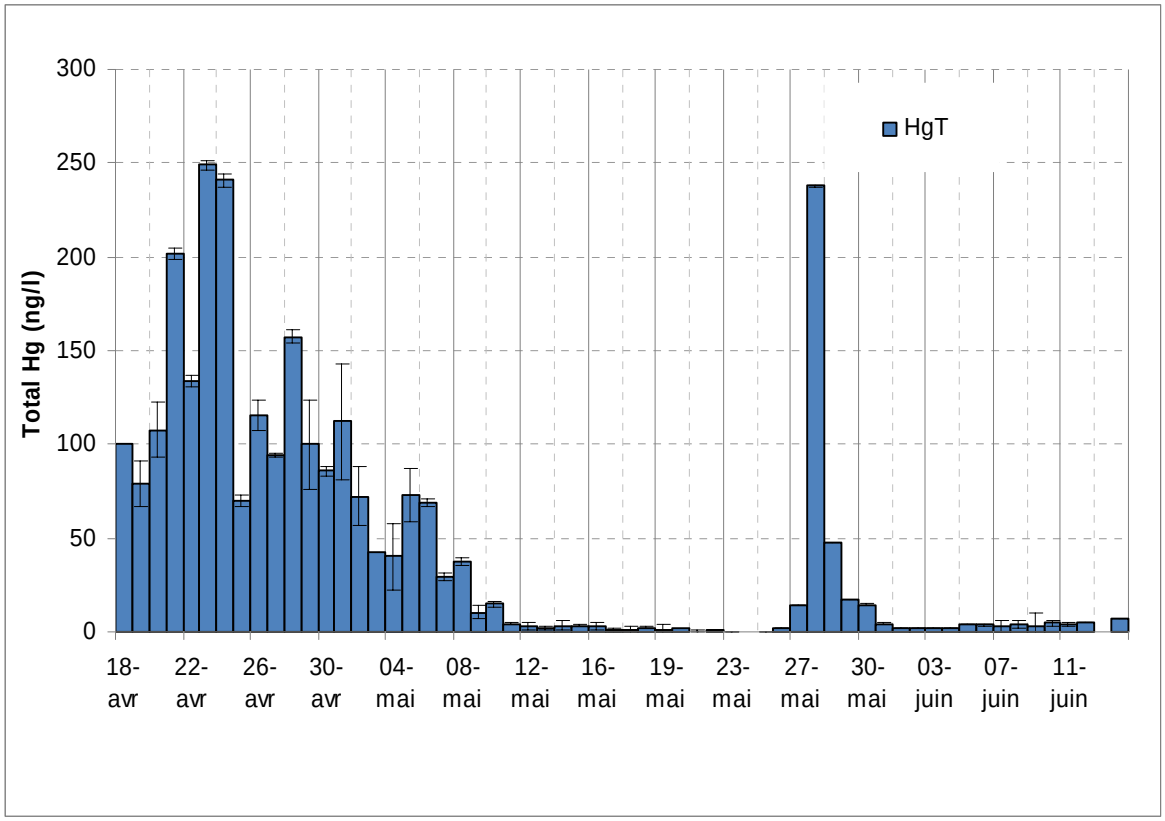


Figure 10.2: Total mercury (ng.L^{-1} of melted snow) measured in Ny-Ålesund snow in Spring 2007.

Figure 10.2 shows that the mercury content in snow increased to high mercury levels until 250 ng.L^{-1} of total mercury between 22nd and 26th of April and between 27th and the 30th or may.

These high levels correspond to Atmospheric mercury deposition events previously observed in gaseous elemental mercury monitoring (Figure 10.1).

After an AMDE the concentration of mercury in snow stay high within few days.

Without these events, total mercury concentrations in snow were low like visible in the period ranging from the 10th to the 27th of May and after the 30th of May.

It should be noticed that AMDE occurred on the snow but also on the sea.

4 Presentation of the Total Mercury Concentrations by size fraction depending on date

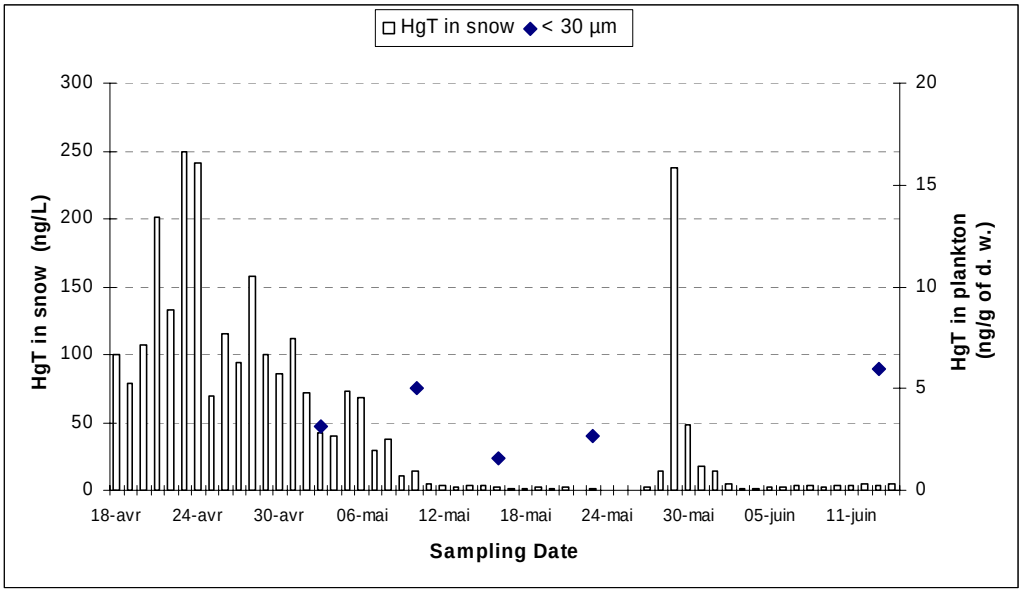


Figure 10.3: HgT concentration in plankton size fraction < 30 µm

Figure 10.3 presents total mercury measurements in plankton for size fraction < 30 µm superposed to mercury measurements in snow. There were no measurements on the 25th of April, on the 30th of May and the 6th of June because the amount of plankton collected for this fraction was too small at these dates. The last value was 6 ng/g of d. w. on the 13th of June.

Mercury values measured in plankton started at 3,15 ng/g of dry weight, then raised 5 ng/g of d. w., to decrease to 1,6 ng/g of d. w., followed by a value of 2,7 ng.g of d.w.

For this size fraction < 30µm, the variation observed in mercury content were low and did not seem to be link to mercury content in snow.

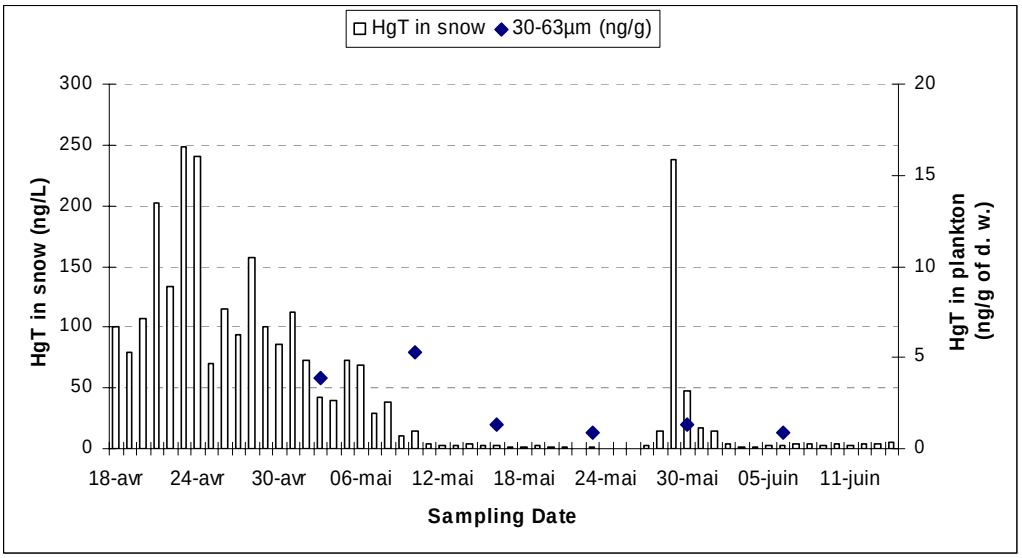


Figure 10.4: HgT concentration in plankton size fraction 30-63 µm

Figure 10.4 presents total mercury measurements in plankton for size fraction 30-63 µm superposed to mercury measurements in snow. There were no measurements on the 25th of April and on 13th of June because the amount of plankton collected for this fraction was too small at these dates.

Mercury values measured in plankton started at 3,9 ng/g of dry weight on the 3rd of May, the next week the value measured was 5,3 ng.g of d.w. The week after it decrease to 1,3 ng/g of d.w. to raised 0,9 ng/g of d.w. on the 24th of May. The last two values measured were 0,9 and 1,3 ng.g of dry weight.

As already observed for the smaller size fraction, the variations of the mercury content in plankton were low and no correlation between mercury in snow and mercury in plankton was noticeable on this figure.

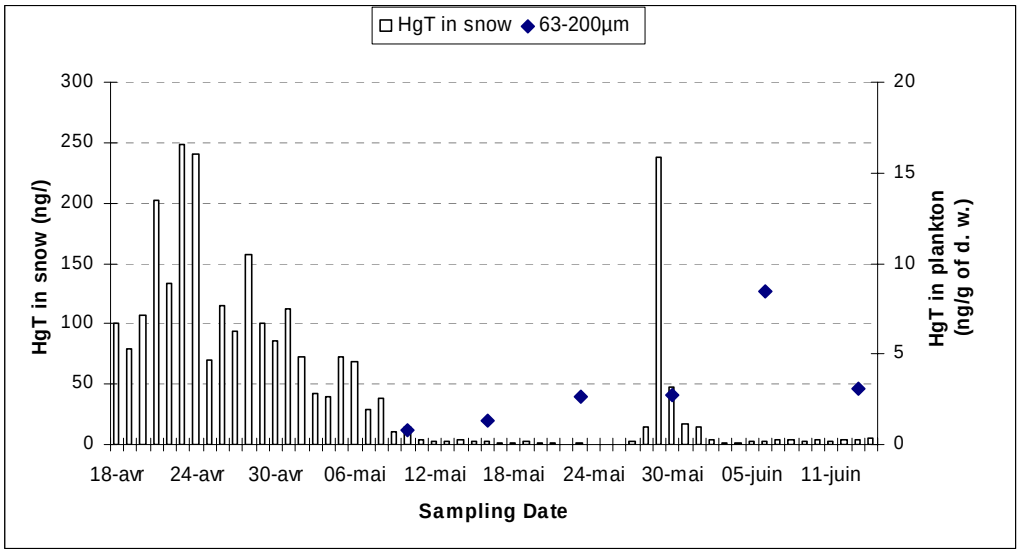


Figure 10. 5: HgT concentration in plankton size fraction 63-200 µm

Figure 10.5 presents total mercury measurements in plankton for size fraction 63-200 µm superposed to mercury measurements in snow. There were no measurements on the 25th of April and the 3rd of May because the amount of plankton collected for this fraction was too small at these dates.

Mercury values measured in plankton was 0,8 to 8,5 ng/g of dry weight for the 10th of May, then it increase slowly until 2,6 ng/g of d. w. for the 23rd of May, and stay stable until an increase at 8,5 ng/g of d. w. on the 6th of June, the last measurement indicates a mercury content of 3,1 ng/g of d. w. for the 13th of June.

For this size fraction, the variation observed are higher, specially on the 6th of June but no correlation with the mercury content in the snow could be underline.

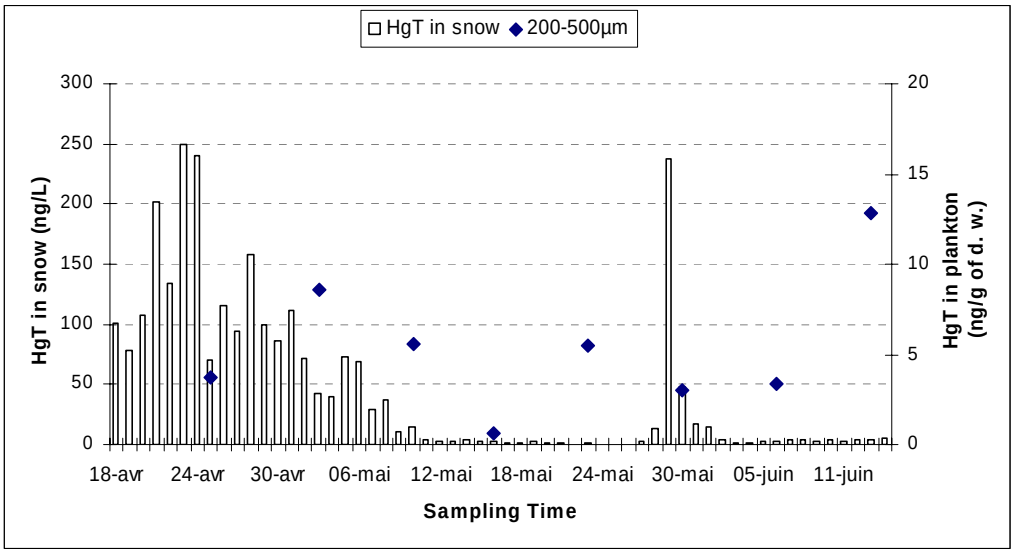


Figure 10. 6: HgT concentration in plankton size fraction 200-500 µm

Figure 10.6 presents total mercury measurements in plankton for size fraction 200-500 µm superposed to mercury measurements in snow. For this planktonic size fraction there were samples for every sampling date.

Mercury values measured in plankton started at 3,7 ng/g of dry weight, the next week it increases at 8,6 ng/g of d.w , before decreasing slowly to raise 0.6 ng/g of d.w. on the 16th of May. The week after the mercury content incease again at 5,5 ng/g of d.w., it stays around 3 ng/g of d. w. on the 30th of May and 6th of June, and raises a maximum of 12,8 ng/g of d.w. on the 13th of May.

For this size fraction, some variation in the mercury content are visible, but their correlation with mercury content in snow is still not clear even if we could observe a minimum in the mercury content in plankton during the period of minimum level of mercury in snow (16th of May).

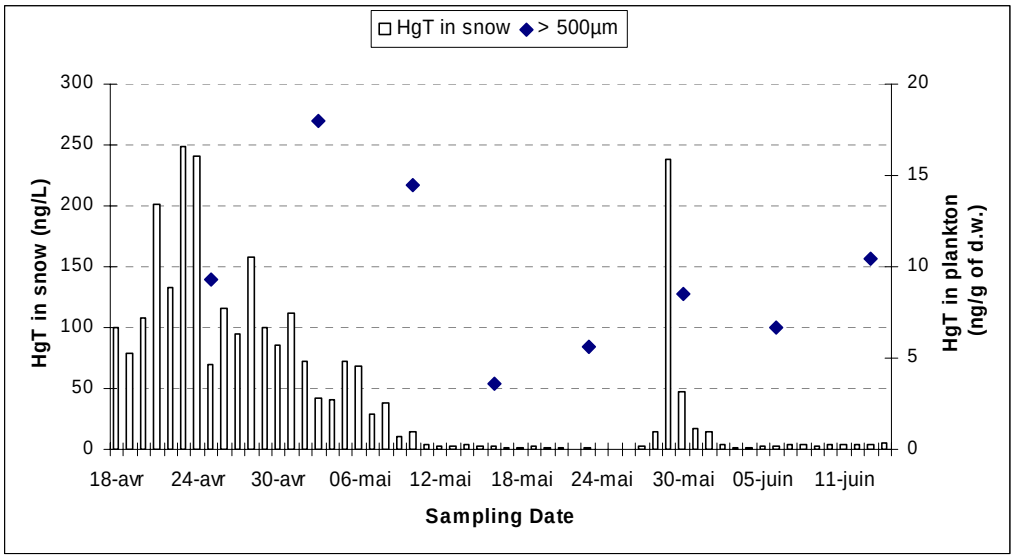


Figure 10. 7: HgT concentration in plankton size fraction > 500 µm

Figure 10.7 presents total mercury measurements in plankton for size fraction > 500 µm superposed to mercury measurements in snow. For this planktonic size fraction there were samples for every sampling date.

Mercury values measured in plankton started higher than for the other fractions at 9,3 ng/g of dry weight on the 25th of April, then it increases at a maximum of 18 ng/g of d. w. on the next week. The values measured for the two following weeks decreases to range 3,6 ng/g of d. w. on the 16th of May. Another increase which raised mercury value of 8,5 ng/g of d. w. was finally observed on the 30th of May. Finally, the values measured for the 6th and the 13th of June were respectively 6,7 and 11 ng/g of dry weight.

For this fraction too, the variation in mercury content are more visible but are still not correlated with mercury content in snow.

5 Presentation Total Mercury Concentration in plankton depending on size fraction

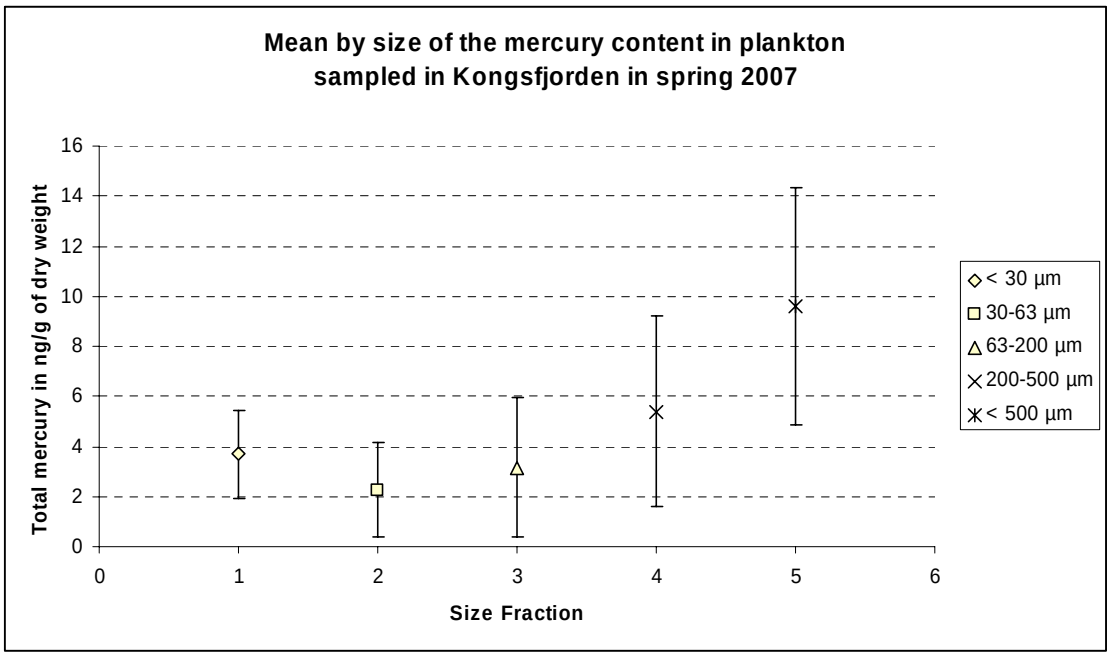


Figure 10.8: Mean of mercury concentrations in plankton depending on size fraction

Figure 10.8 presents the mean of total mercury concentration in the different planktonic samples, collected during spring 2007, sorted by size fraction. The error bar corresponds to the standard deviation between the measured obtained.

This figure shows that despite a big variation between the mercury content measured in each fraction, the total mercury measurements seems higher in the bigger size fractions than in the smaller ones.

6 Discussion on the mercury level in planktonic samples

Few studies about mercury content in plankton from Arctic exist and they were performed in the nineties. For example Atwell et al analysed in 1998 samples collected in summer 1988 and 1990 in north of the Baffin Sea close to Devon Island. The levels measured in these samples ranged from 60 to 100 ng/g of dry weight.

Joiris et al. 1997, sampled between the Barents Sea and Spitzbergen in July and August 1991 and found total mercury levels in plankton of 90 ng of HgT/ g of dry weight.

Our measurements ranged from 0,8 to 18 ng/g of dry weight that is ten fold lower than the levels found in the two previous studies.

Two other studies proposed measurements on planktonic samples. Campbell et al (2005) sampled in may 1998 also in the north of the Baffin Sea and reported levels between 3 and 25 ng/g of wet weight.

A Norwegian study performed by Jaeger et al. 2007 in summer 2005 and 2006 indicated level lower than the detection limit of 10 ng/g of wet weight.

In this case, as the literature presented results expressed in µg/g of wet weight it is difficult to compare with our results. But in assuming then wet weight is heavier than dry weight we could postulate that the levels of these two studies are probably lower than ours.

Finally in all the studies coming from the literature the samples were harvested in summer and not in spring as ours so it could maybe not be comparable.

Figure 10.8 represent all the data sorted by size. We can see that the levels in the three smaller size (<30µm; 30-63 µm and 63-200 µm) are in the same range. Then for the two bigger ones (200-500 µm and > 500 µm) there's an increase in the mercury levels.

These results are consistent with a bio amplification process observed with a pollutant like mercury. But to confirm this hypothesis the exact composition of each size fraction needed to be known as the structure of the food web.

7 Conclusions and perspectives on the mercury levels in plankton

These data remain incomplete and are preliminary ones but as few ones exists on this subject they are still interesting. They permit to see that the levels of mercury in planktonic samples in spring in Ny-Ålesund were very low, lower than reported in the literature. Moreover methylmercury could not be measured because of these total mercury low levels.

The measurements did not show a correlation between the mercury content in plankton and the mercury deposited on snow.

But to better understand if a link exist between AMDE and mercury content in plankton, another sampling campaign should be done in Kongsförden with a higher sampling frequency (every day for example) and replicates for each size fraction. Finally the campaign should be longer than this one (start before and stop after spring) to see if some variation in the mercury concentrations in plankton could be influenced by the AMDE.

Moreover our measurements concerned size fraction and not isolated planktonic species in each sample fraction, this could not be done because we were in a preliminary work. But a complete study with separation and identification of the different species could also gave interesting data in this subject.

Chapter 11: Total and methyl mercury measurements in polar non marine biofilms

1 Introduction of the chapter

The first aim of this study was to measure total mercury and methylmercury concentrations in biofilms sampled in Arctic and Antarctica. As microbial biofilms are known to be able of binding significant quantities of metallic ions under natural conditions (Ferris, 1989), we wanted to have an idea of the mercury and methylmercury content in such biological samples.

The second goal of this work was to see if biofilms could concentrate or methylate inorganic mercury. This was done by exposing a natural biofilm during 48 hours to different concentration of mercury (0, 50, 200, 500, 1000, 5000 ng/L). This biofilm was collected in Byers Peninsula, South Shetlands Islands, Antarctica and the experiment was done in acid-washed glass vessels, the volume assay was 50 mL.

After incubation with mercury, microbial mats were washed with water and stored frozen until total mercury and methyl mercury analysis.

The following parts presents the details of the samples and the results obtained for these two experiments.

2 Details of the native biofilms

The details of the samples are presented in the table 11.1.

	SAMPLE NUMBER	LOCALISATION	LATITUDE	TAXONOMIC COMPOSITION	NOTES
ANTARCTIC	1	Hope Bay, Trinity peninsula Lake Boekella	64°S	• Not determined	• Close to Argentinean base • probably contaminated by debris or petrol from the base
	2	Mac Murdo Ice shelf Casten pond	78°S	• Not determined	• very old (centuries to millenia) • only atmospheric deposition
	3	Mac Murdo Ice Shelf Black dot pond	78°S	• Mainly cyanobacteria	• fresh material
	4	Mac Murdo Ice Shelf Fog Horne pond	78°S	• Monospecific community of cyanobacteria (Nostoc)	• quite young
	5	Mac Murdo Ice Shelf Conophyton pond	78°S	• Mainly cyanobacteria	• probably old (decennia, centuries)
	6	Deception Island Lake Nido	63°S	• Mainly cyanobacteria with diatoms	• last volcanic eruption in the 1960s
	7	Mac Murdo Ice Shelf Casten Pond	78°S	• Mainly cyanobacteria	• younger than sample 2
	11	Byers Peninsula Lake Suroeste	62°S	• High diatoms content and cyanobacteria	• young orange mat
	12	Byers Peninsula Lake Suroeste	62°S	• Mainly cyanobacteria	• young purple mat
	A	Byers Peninsula Livingstone Island	62°S	• Lichen	• Hill summit • far from human activity
	B	Byers Peninsula Livingstone Island	62°S	• Moss	• Hill summit • far from human activity
	C	Byers Peninsula Livingstone Island	62°S	• Soil	• Hill summit • far from human activity
ARCTIC	8	Ellesmere Island Lake Ward Hunt	83°N	• Mainly cyanobacteria	• close to the camp where north pole expedition leave
	9	Ellesmere Island Lake C1	82°N	• Mainly cyanobacteria	• far from human activity
	10	Ellesmere Island Lake C1	82°N	• Mainly cyanobacteria	• far from human activity

Table 11.1: Summary of the morphological characteristic of the mats samples

Table 11.1 indicates that most of the biofilms were sampled on different ponds or lakes and are constituted by cyanobacteria except for 2 samples which are not determined (1 and 2).

3 Results for mercury and methylmercury measurements

Origin	Sample Number	Localization	[HgT] (ng/g of dry weight)	[MeHg] (ng/g of dry weight)	% MeHg
Antarctic	1	Trinity Peninsula	399	5,45 ± 0,07	1,37
	2	Mac Murdo Ice Shelf	72	0,38 ± 0,02	0,52
	3	Mac Murdo Ice Shelf	14	1,05 ± 0,01	7,49
	4	Mac Murdo Ice Shelf	43	2,99 ± 0,19	6,96
	5	Mac Murdo Ice Shelf	80	2,1 ± 0,08	2,62
	6	Deception Island	29	2,92 ± 0,05	10,09
	7	Mac Murdo Ice Shelf	18	0,82 ± 0,01	4,54
	11	Byers peninsula	90	2,05 ± 0,04	2,28
	12	Byers peninsula	69	3 ± 0,03	4,35
Arctic	8	Lake Ward Hunt	55	1 ± 0,01	1,82
	9	Ellesmere Island	30	0,3 ± 0,06	1
	10	Ellesmere Island	65	1,3 ± 0,13	1,99

Table 11.2 : Total Mercury concentration (HgT), Methyl-mercury concentration (MeHg) and Methylmercury percentage (% MeHg) in 10 mats from Arctic and Antarctic. Methylmercury percentage was calculated as (MeHg/HgT)*100.

Table 11.2 presents the results of total mercury and methylmercury measurements in the biofilm samples.

The values obtained, except for the sample 1, ranged from 14 to 90 ng/g of dry weight of total mercury in the Antarctic samples and from 30 to 65 ng/g of dry weight for the Arctic ones. The sample 1 contained 399 ng total mercury/g of dry weight.

Table 11.2 also presents the value measured for methylmercury. Measurements ranged from 0,38 ± 0,02 ng/g of dry weight to 5,45 ± 0.07 ng/g of dry weight for Antarctic samples and between 0,3 ± 0,06 to 1,3 ± 0,13 ng/g of dry weight for Arctic samples.

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The percentage of methylmercury compared to mercury ranged from 0,52 % to 10,09 % for the Antarctic samples and from 1 to 1,99 % for the Arctic ones.

Sample code	Type	Localisation	[HgT] (ng/g of dry weight)
A	Lichen	Antarctic Byers peninsula	163
B	Moss		83
C	Soil		31

Table 11. 3: **Total mercury concentration in Lichen, Moss and Soil samples from Byers Peninsula, Antarctic.**

Table 11.3 presents the results of total mercury concentrations in lichens, moss and soil. The levels were respectively 163, 83 and 31 ng/g of dry Weight.

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4 Results for exposed biofilms

Exposed concentration in ng/L of $HgCl_2$	HgT in ng/g of dry weight (mean, n=2)		MeHg in ng/g of dry weight (mean, n=2)		% de MeHg (mean, n=2)	
0	33,5	± 4,95	1,45	± 0,08	4,34	± 0,4
50	31,5	± 2,12	1,66	± 0,03	5,29	± 0,3
200	38,5	± 2,12	1,54	± 0,01	4,02	± 0,2
500	42	± 0,00	2,08	± 0,12	4,96	± 0,3
1000	39	± 4,24	1,46	± 0,04	3,75	± 0,3
5000	74	± 11,31	1,88	± 0,10	2,59	± 0,5

Table 11. 4: Total mercury, methylmercury and methylmercury percentage in a biofilm exposed to mercury.

Table 11.4 presents total mercury, methylmercury and methylmercury percentage measured in the exposed biofilms. The values for total mercury range from 31,5 to 74 ng/g of dry weight.

For methylmercury the measured values were between 1,45 to 2,08 ng/g of dry weight which correspond to percentage of the Total Mercury between 2,59 and 5,29 %.

The total mercury and methylmercury values measured for the exposition concentration of 0 ng/L of mercury (respectively 33,5 ng/g of d. w. and 1.45 ng/g of d. w) corresponds to the basal level of mercury of the mat.

5 Discussion and conclusion

5.1 On native biofilms

5.1.1 Total mercury measurements

In benthic mats from lakes in Antarctica, Bargagli et al 2007 measured mercury concentration ranging from 33 to 108 ng/g of dry weight. The samples analysed were cyanobacterial mats from four location of Victoria land Antarctica (75°S) (Bargagli et al, 2007).

So except for the sample 1 the total mercury values measured in our samples are coherent with the ones found in literature.

To discuss about these data they will be grouped depending on their geography by opposing north Antarctica and south Antarctica.

Samples 2, 3, 4, 5 and 7 came from the south of Antarctica (SA), they were samples in different ponds located on the Mac Murdo Ice Shelf.

Samples 3 and 7 respectively 14 and 18 ng/g of dry weight of HgT gave the lowest levels of mercury, as indicates in Table 1, they came from two ponds on the Mac Murdo Iceshelf ablation zone, and they are constituted of mainly cyanobacteria. They were not subject to human activity. This localisation on a remote environment and far from humans explain the low total mercury levels measured.

The sample 4 came from another pond on the Mac Murdo Iceshelf and was constituted by monospecific of Nostoc cyanobacteria, the HgT value measured was a little higher (43 ng/g of dry weight) maybe this mat was older than samples 3 and 7, or maybe this monosecific community concentrate more mercury than a mix one.

Samples 2 and 5 were sampled in two other ponds on the ice shelf. We measured the highest values (72 and 80 ng/g of dry weight) for HgT. These two mats are older than the other samples from South Antarctica (see Table 4.11) this explain their higher mercury content.

In any case Mac Murdo station is known to be subject to atmospheric mercury depletion events (Brooks et al, 2008) such as Victoria land, this explain that we found non negligible mercury levels, comparable to those observed in in Antarctica by Bargagli et al, 2007.

Samples 1, 6, 11 and 12 came from the north of Antarctic (NA) close to Byers peninsula.

Sample 1 corresponded to a mat sampled in Hope Bay on Trinity peninsula, close to the Argentinean base. It was contaminated by debris and petrol that explains the high mercury content (399 ng/g of dry weight of HgT).

Sample 6 came from a lake in Deception Island where several volcanic eruptions occurred, its mercury content was not high (29 ng/g of dry weight).

Samples 11 and 12 were originated from a lake on Livingstone Island, they gave the higher total mercury values (90 and 69 ng/g of dry weight), they supposed to be not subjected to human influence, only atmospheric deposition. They did not have the same taxonomic composition but both gave high mercury

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value, these samples were maybe older than others, or this area was maybe more subject to mercury deposition.

These observations showed there were not a big difference in mercury content between the north and the south of Antarctica but we can notice that the mercury concentration are a little higher in the North of Antarctica.

Concerning lichen, moss and soils measurements, the mercury values measured corresponded to the typical background values for Antarctic soils, lichens and mosses (Crockett, 1998) and showed the typical distribution pattern: Hg lichen>Hg mosses> Hg soil (Bargagli et al, 2007), indicating a bioconcentration between soil and plants.

5.1.2 Methylmercury measurements and methylmercury percentage

Concerning methylmercury concentration in the biofilms we saw higher levels in the samples from the north of Antarctica than for the south ones (mean value was 6,65 ng/g of dry weight for samples 6,11 and 12 (NA) versus 1,28 ng/g of dry weight for samples 2,3,4,5 and 7 (SA)).

For the sample 1 we found 5,45 ng/g of dry weight of methylmercury which is higher than the other values but this sample was clearly contaminated with a level of total mercury of 399 ng/g of dry weight and if we look the percentage of MeHg it was in the same proportion that for the others biofilms.

The sample 6 from Deception Island gave a methylmercury percentage of 10% which is higher than the others results.

North of Antarctic is close to the Weddell sea which is a location known to be subject to frost flowers which are phenomenon correlated to atmospheric depletion events (Kaleschke et al, 2004).

The methylmercury concentration in the Arctic samples ranged from 0,3 to 1,3 ng/g of dry weight which represented less than 2% of the total mercury content. This methylmercury percentage is lower than the one observed for Antarctic samples.

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Desrosier et al, 2006, found total mercury concentrations in periphyton biofilm of unperturbed lakes from less north samples (47-50°N) between 42 to 271 ng/g of dry weight and MeHg 3 to 55 ng/g of dry weight.

We could notice that biofilms from polar regions didn't contain more mercury than ones from area non subjected to mercury deposition.

5.1.3 Exposed biofilms

Figure 11.4 presents total mercury and methyl mercury measured in the exposed biofilms.

The results shows for low exposition concentrations like 50 ng/L total mercury value in the mat was equal to the basal value. For an exposition concentration of 200 ng/L the total mercury content increased of 5 ng/L, for 500 and 100 ng/L the increase was about 10 ng/L.

For an exposition concentration of 5000 ng/L total mercury content increase by a factor 2 compare to the basal level (74 ng/L versus 33,5 ng/L).

These results suggested that at high concentrations close to (5000 ng/L of $HgCl_2$) inorganic mercury could diffused through the biofilm in 48 hours and accumulates inside it.

In this experiment methylmercury levels stayed low between 1,45 to 2,08 ng/g of dry weight and methylmercury percentage were between 2,59 to 5,29 % without an increase with the exposed concentration indicating there's no more methylmercury in a mat exposed to high mercury concentration.

This lead to think that biofilms exposed to environmental amount of inorganic mercury did not methylate it in 50 hours.

Observation of a storage of mercury in biofilms was coherent with the current knowledge about biofilms. They are known to be able to react with metals (Ferris et al. 1989).

In this experiment the idea was to see if environmental amount of mercury in the range of hundreds ng/L could concentrate in biofilms in 48hous. This phenomenon was not observed by this experiment.

6 Conclusion

This study permits to observe that total mercury levels in native biofilms from Arctic and Antarctica were low between 30 to 90 ng/g except for one sample which was clearly contaminated (399 ng/g) but this corroborate the idea that biofilms exposed to high level of mercury could concentrate it. No difference was underlined between the mercury levels in mats from Arctic than from Antarctic.

Mercury was also present in lichen, moss, and soil sampled in Antarctic with higher concentrations in lichens than in moss than in soils (respectively 163; 63 and 31 ng/g of dry weight) this observation indicated a concentration of mercury in the plant compared to the soil. Moreover, lichens and mosses may also collect metals from the atmospheric aerosol (Basile et al. 2001, Genoni et al. 2000), this indicates the increase of mercury concentration emphasized in table 11.4 could also derive from the atmosphere.

Methylmercury levels measured in native mats form both Arctic and Antarctica indicated that a part of the mercury was under the form of methylmercury. The highest methylmercury content was for an obviously contaminated mat, but the methylmercury percentage stayed in the range of the other samples indicating there were no more methylmercury in mats collected in a contaminated area.

These results indicates that mercury could be found in both Artic and Antarctic mats, but the levels measured stayed low, in the range of the ones found in remote environment (Krishna et al. 2002).

Globally methylmercury concentrations in this exposition experiment were low but detectable and represented about 2% of the total mercury content.

The origin of this methylmercury is still unclear. But these results corroborate the idea of a methylation in the environment rather than in the mats themselves. The results obtained in chapter 8 showed that yeasts cells could store methylmercury without demethylation this should also be the case for biofilms. In that case this kind of bacterial structure could be a sort of trap for methylmercury in the environment.

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Exposition of biofilms to mercury at environmental levels of mercury during 48 hours did not underline a clear storage of inorganic mercury at these concentrations (hundreds of ng/L) but the phenomenon seemed to occur at higher ones (thousands of ng/L). It could be interesting to perform this exposition experiment on a slight longer time period (few days) and with higher concentration to better understand mercury storage in such biological material.

In this study, it was not possible to analyse organic C concentrations and to evaluate the age of the biofilms, despite this information could help to understand mercury accumulation.

So in perspective of this work, some other sampling should be done with more chemistry analysis in order to compare the chemical composition of the mats and the mercury and methylmercury measurements.

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Chapter 12: Conclusions and perspectives

1 In French

1.1 Conclusions

Ce travail de thèse avait pour but de répondre à certaines questions concernant les micro-organismes de la neige et le mercure en arctique. Ces questions étaient les suivantes :

- Y-a-t-il des micro organismes dans la neige ?
- Si oui lesquels ? Et peuvent-ils survivre dans ce milieu au printemps ?
- Si des micro organismes sont présents, peuvent-ils réagir avec le mercure déposé lors des AMDE ?
- Et donc finalement, les micro organismes de la neige jouent-ils un rôle dans le cycle du mercure en zone polaire ?

Les premiers travaux menés sur l'isolement et le caractérisation des souches, illustrés par la publication Amato et al, 2007 ont montré que la neige arctique renfermait un nombre non négligeable de microorganismes. Dix bactéries ont pu être isolées et identifiées à partir de cette neige, huit d'entre elles correspondent à des souches déjà retrouvées dans des environnements froids, et 2 d'entre elles n'ont pu être totalement identifiées ce qui laisse à penser à de nouvelles souches encore inconnues.

Ces dix souches bactériennes ont été capables de dégrader des acides organiques présents dans la neige, et certaines sont capables de se développer à 5°C. Ces observations indiquent que ces souches bactériennes peuvent probablement se développer au printemps au moment de la fonte de la neige.

Trois souches de levures ont également été isolées de la neige arctique, l'une d'entre elle est une levure rare répertorié dans une collection de levure, les deux autres appartiennent à un genre connu mais l'une d'entre elle n'avait jamais été répertoriée et a été ajoutée à la base de données de CBS.

Ces premiers travaux ont donc permis de répondre aux premières questions posées.

La suite du travail a permis de mettre en évidence la présence de gène de résistance au mercure chez deux des souches arctiques. Ce qui indique que ces

deux bactéries sont capables de réduire le mercure divalent en mercure élémentaire.

De plus lors d'une expérience d'interaction entre du mercure divalent et des levures, on a pu observer une disparition du mercure inorganique indiquant sa probable volatilisation par les levures.

Ces deux résultats laissent à penser que les micro organismes pourraient participer à la volatilisation du mercure divalent en mercure élémentaire.

En effet ce phénomène est observé dans la neige, après un AMDE, une bonne partie du mercure divalent est re-volatilisé dans les deux jours, ce processus semble être majoritairement photo chimique mais on peut penser que les micro organismes jouent un petit rôle dans ces mécanismes.

Dans la continuité de ce travail, les manipulations d'interactions effectuées entre le méthylmercure et les levures ont montré que celles ci étaient capables de stocker le méthylmercure sans le dé-méthyler. Ce résultat indique que certains micro organismes peuvent agir comme des éponges à méthylmercure et que la bio-amplification du mercure chez les êtres vivants commence certainement dès ce niveau trophique. Dans tous les cas, une fois formé dans l'environnement, le méthylmercure sera à priori absorbé rapidement par les cellules vivantes.

Les travaux effectués pendant cette thèse ont également concerné des mesures de mercure total et de méthylmercure dans des biofilms arctiques et antarctiques ainsi que dans des échantillons biologiques (lichen, mousse et sol). Les résultats obtenus pour les biofilms ont montré que ces structures bactériennes renfermaient du mercure a des niveaux relativement bas sauf pour un échantillon clairement contaminés. Les résultats obtenus pour les échantillons terrestres ont montré une bioamplification du mercure entre le sol, les mousses et les lichens. La part du mercure organique dans toutes ces mesures est de 2% en moyenne.

Lors d'une expérience d'exposition d'un biofilm au mercure pendant 48 heures nous n'avons pas observé d'augmentation du taux de méthylmercure dans le biofilm ce qui indique qu'apparemment sur le laps de temps, et avec les concentrations testées le biofilm n'était pas capable de méthyler le mercure.

Par contre lors de cette expérience il a semblé que pour une concentration de l'ordre de 5 µg/L de mercure inorganique le biofilm avait stocké une partie du mercure.

Ces résultats laissent à penser que ce type de structure bactérienne est capable de stocker du mercure inorganique et du méthylmercure. Par contre la méthylation ne semble pas se produire au niveau du biofilm lui-même, on peut penser que le biofilm se conduit comme des cellules de levures, c'est à dire qu'il piège le méthylmercure formé dans l'environnement mais qu'il n'est pas capable de méthyler le mercure inorganique.

Finalement les travaux effectués ont permis de mesurer des niveaux de mercure total dans du plancton arctique. Les niveaux sont très bas et les variations observées ne paraissent pas corrélées avec les AMDE, mais cette observation demande à être confirmée par une campagne d'échantillonnage plus importante.

La question de la méthylation du mercure en arctique reste donc peu comprise, bien que la piste d'une méthylation dans les sédiments marins soit la plus plausible.

Nos travaux ont toutefois permis de montrer que ce phénomène ne s'observait pas dans des cultures pures de levures, ni dans des biofilms exposés au mercure inorganique et que les niveaux de méthylmercure dans le plancton n'étaient pas détectables.

1.2 Perspectives

Finalement ce travail a ouvert de nombreuses perspectives.

Tout d'abord au niveau microbiologique, nous avons testé 2 milieux de culture (R2 et TS) pour isoler des souches bactériennes de la neige. Il serait intéressant de tester d'autres milieux par exemple, comme la neige collectée provient d'une zone côtière, un milieu de culture pour bactéries marines pourrait permettre l'isolement d'autres souches.

De plus sur les 2 souches isolées non identifiées totalement, un séquençage de l'ARN 23S permettrait de terminer l'identification et donc de voir si ces 2 souches ont déjà été isolées ou sont totalement nouvelles.

Durant cette thèse, nous avons travaillé avec des méthodes microbiologiques basées sur les bactéries cultivables, mais des méthodes moléculaires d'études de biotopes existent, comme pour l'étude des sols par exemple, elles ont pour principe d'extraire et d'amplifier l'ADN de la matrice à étudier afin d'avoir une meilleure idées de la diversité bactérienne présente sur un site. On peut donc citer la SSCP*, la RFLP**, les DGGE*** ou la métagénomique, etc. Une thèse sur la métagénomique de la neige a d'ailleurs démarré en 2007 au LGGE portée par Catherine Larose.

Ce type d'approche pourrait fournir des informations permettant d'évaluer la biomasse microbienne dans la neige.

Finalement notre travail s'est focalisé sur les levures et les bactéries mais des champignons et des algues sont aussi capables de vivre dans ce genre d'environnements, il serait également intéressant de les rechercher pour pouvoir ultérieurement étudier leurs comportements face au mercure.

Nos travaux sur les bactéries et le mercure ont montré que certaines souches possédaient des gènes de résistance au mercure, elles sont donc à priori capables de réduire le mercure divalent. Cette propriété mériterait d'être testée par une manipulation de contrôle (test de la volatilisation du mercure dans des cultures pure de bactéries enrichie en mercure inorganique par exemple). Ce type de manipulation pourrait également être réalisé avec les souches de levures afin de confirmer les résultats que nous avons observés dans la manipulation d'interaction.

En ce qui concerne cette manipulation, les nombreux ajustements présentés dans les perspectives des chapitres 8 et 9 seront nécessaires afin de garantir la qualité des résultats obtenus.

Au niveau des biofilms, nous avons constaté qu'ils pouvaient stocker du mercure inorganique et du méthylmercure sans voir de méthylation au sein même du biofilm lors d'une expérience d'exposition au mercure. Ce point mériterait d'être approfondi par d'autres expériences d'exposition avec des isotopes par exemple ce qui permettrait de mieux suivre le devenir du mercure.

*Single Strand Conformation Polymorphism

** Restriction Fragment Length Polymorphism

*** Denaturing gradient gel electrophoresis

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De plus une meilleure caractérisation des biofilms du point de vue chimique (composition exacte, analyses des taux de carbone, d'azote, etc.) permettrait peut être de mieux comprendre le stockage du mercure par ce type de structure.

Il serait finalement intéressant d'échantillonner à nouveau des biofilms natifs et de mesurer leurs taux de mercure, surtout au niveau de l'arctique ou peu de données existent.

La dernière partie de ce travail portait sur la mesure des taux de mercure dans des échantillons de plancton arctique. Les résultats n'ont pas montré d'influence claire des AMDE sur les taux de mercure mesurés dans les différentes fractions mais pour confirmer cette observation une étude plus complète, avec des échantillonnage plus fréquents et plus étalés dans le temps, serait nécessaire pour pouvoir établir ou non un lien avec les AMDE.

L'étude des micro organismes dans le cycle du mercure en zone polaire reste donc une piste intéressante car les résultats obtenus ont montré qu'ils pouvaient réagir avec les quantités environnementales de mercure.

De nombreux points restent à approfondir mais ces travaux, les premiers sur ce type de problématique au LGGE, ont montré que la composante microbiologique pouvait jouer un rôle dans le cycle du mercure en zone polaire.

2 In English

2.1 Conclusions

The aim of the work performed during the PhD was to answer to several questions about microorganisms in Arctic and mercury, which were:

- Are there microorganisms in snow?
- If yes, which ones and are they able to live in this environment during spring?
- And finally are they able to react with amount of mercury deposited during AMDE with the idea of a possible methylation in snow.
- This work was performed with the idea to see if microorganisms play a role in the mercury cycle in polar areas.

The first work performed on isolation and characterisation of bacteria isolated from snow in Svalbard was illustrated by the publication Amato et al, 2007. This showed that arctic snow could contain microorganisms. Ten bacterial strains were isolated and identified from this snow. Eight of them corresponded to bacterial strains already found in extreme environment and the last two were not totally identified, which indicates they are probably new strains.

These ten bacterial strains were able to degrade organic acids naturally present in snow, and some of them were able to grow at 5°C. These observations permit to think that these strains could grow in spring during snowmelt.

Three yeasts strains were also isolated from Arctic snow, one of them is a rare specimen only known in a yeast collection, the two other belongs to a known genus but one of them never been reported yet and was added to the yeast data base of CBS.

This previous work permits to answer to the first questions.

After this first step, the presence of mercury resistance gene in the bacterial strains isolated from snow was detected among two strains. This indicates that these bacteria are able to reduce divalent mercury to elemental mercury. Moreover, during an interaction experiment with mercury and yeast, we saw a disappearance of divalent mercury from the medium, which indicates its probable volatilization by yeast.

These two results permits to say that microorganisms in snow could participate to the volatilization of divalent mercury in elemental mercury.

Indeed this phenomenon is already observed in snow. After an AMDE, a big part of the divalent mercury deposited is re-emitted to the atmosphere within two days. This process seemed to be globally driven by photochemistry but microorganisms could have a small role in it.

Interaction experiments between methylmercury and yeast showed they were able to store methylmercury without de-methylate it. This result indicates that some microorganisms could act as methylmercury sinks and that bio magnification of mercury in food webs start at this trophic level. In any case, once formed in the environment, methylmercury will be probably quickly absorbed by living cells.

During this PhD, some mercury and methylmercury measurements in biofilms and biological samples collected in Arctic and Antarctic were also performed. The results showed that these bacterial structures were contaminated by mercury but with relatively low levels except for a clearly contaminated sample. IN addition measurements performed in lichen, moss and soil showed a pattern of bioaccumulation of mercury.

The part of the mercury under organic form in biofilms was 2% in mean.

During an exposition of a biofilm to mercury within two days, no increase of the methylmercury content in the biofilm was observed, this indicates that on this time period and with mercury amount tested, the biofilm was not able to methylate mercury.

In opposition, during this experiment it was seen that for a 5 µg/L mercury concentration, the biofilm stored a part of the mercury. These results led to think that this kind of bacterial structure was able to store inorganic mercury and methylmercury. Biofilms were thought to be able to trap methylmercury formed in the environment such as yeast cells did, but were not able to directly methylate mercury.

To finish, the last part of the work permitted to measure mercury content in planktonic samples collected during spring 2007 in Svalbard. The level

measured were very low but seemed to vary in correlation with AMDE. But this observation needs to be confirmed with a bigger sampling campaign.

The phenomenon leading to mercury methylation in Arctic stays unclear even if the idea of a methylation by sulfato-reductant bacteria in anoxic sediments seemed the most probable

Our work permits to show that mercury methylation was not observed in pure yeast culture; neither in exposed biofilms and that methylmercury levels were not detectable in plankton in Arctic.

2.2 Perspectives

The work performed during the PhD opened a lot of new perspectives.

To start, on the microbiological point of view, we chose to focus on cultivable microorganisms in snow and we tested two growth media to isolate them: TS and R2. R2 and TS are common media already used to isolate microorganisms from cold environment because they are poor in nutriment and permits to recover species used to live in extreme conditions.

An interesting perspective could be to test other isolation medium such as saline medium because the sampling site is a close to the see and the snow composition should be influenced by marine ions.

During this work we decide to focus on cultivable bacteria. This kind of method permits to recover only a small part of the microorganisms present in the environment, between 1 and 10%. But it was useful for us because the interest was to test the interaction of these microorganisms with mercury.

Some other methods, based on a direct extraction of DNA, exist to investigate the microbial content of a media like snow. These kinds of methods are used to study the profile of all the bacterial community belonging to a kind of environment. The mains method used to study microbial ecology are SSCP*, RFLP**, l DGGE***, metagenomic study.

*Single Strand Conformation Polymorphism

** Restriction Fragment Length Polymorphism

*** Denaturing gradient gel electrophoresis

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These types of method are commonly used on ecosystems like soil, or marine biota.

It could be very interesting to apply them on snow to better known and understand the dynamic of bacterial communities in this habitat.

A PhD start on this subject in the LGGE and is performed by Catherine Larose ("Metagenomic study of the microbial content in Arctic snow").

This type of research is a good complement to cultivable microbiological methods and will give some new information about bacterial community in snow.

Some annual studies of the microbial content and ecology in snow could permit to evaluate the part taken by microorganisms in term of biomass in this kind of environment.

Moreover, as they were the first species isolated from the snow in 2004, our work was focused on yeast and bacteria but it is known that algae and fungi could be found in snow and their behaviour with mercury could be an interesting perspective.

The work performed on bacteria and mercury showed that some strains carried resistance gene against mercury, so they thought to be able to reduce divalent mercury to elemental mercury. This property needs to be test by a control experiment (i.e. monitoring mercury volatilization in pure culture of bacteria). This kind of experiment could also be performed with yeast strains to confirm the results observed in the interaction experiment.

Regarding this interaction experiment, there's still a lot of work to do as presented in the perspectives of chapter 8 and 9 to assess a good quality in the results.

Concerning biofilms, it was shown they were able to store inorganic mercury and methylmercury. We did not see any methylation process during an exposition of the biofilms to inorganic mercury. But this type of experiment needs improvement like using isotopes for example to better understand the fate of mercury in such biological samples.

Moreover a better chemical characterization of the biofilm could also help to understand the storage of mercury by this structure.

In any case it could be interesting to sample again this type of bacterial structure, especially in Arctic were few data exists.

The last part of the work was focused on total mercury measurements in planktonic samples. The results showed no impact of the AMDE on the mercury content in plankton. But this result needs to be confirmed by a bigger study including a high frequency for sampling and a sampling campaign covering spring and summer. This could permits to establish or not a link with AMDE.

To conclude the study of microorganisms in the mercury cycle in polar area stays an interesting challenge. The results obtained during this PhD showed that microorganisms could react with mercury in environmental levels. A lot of points need to be improved but this work, the first one on this kind of subject in the LGGE, shows that microbiological component could play a role in mercury cycle in polar area.

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Ressources électroniques :

FDA : www.fda.gov

US- EPA: www.epa.gov

APPENDIX

Association Bernard Gregory
www.abg.asso.fr

Valorisation des compétences des docteurs
« un nouveau chapitre de la thèse ® »

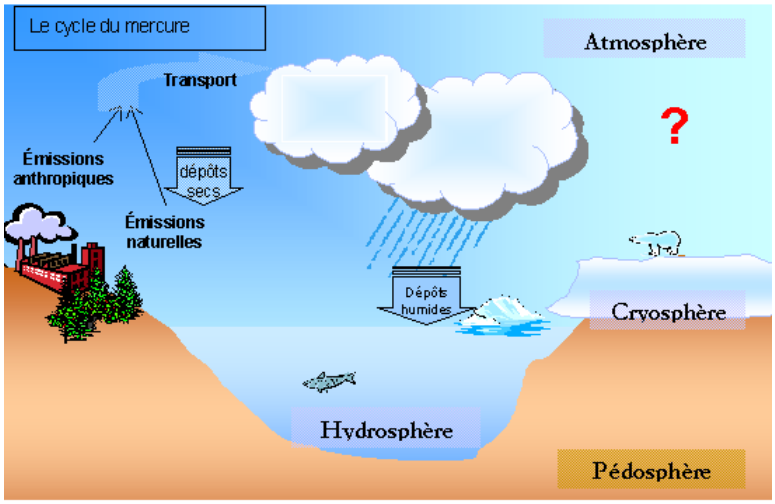
Raphaëlle Hennebelle

Ecole doctorale Terre, Univers, Environnement

Université Joseph Fourier

Nom du "mentor" : Jean-Christophe Klein

Les bactéries et le cycle du mercure en zone polaire



Date probable de présentation orale du « NCT » : Octobre 2008

Sujet académique de la thèse : Etude de la composante microbiologique dans le cycle du mercure en zone polaire.

Nom des directeurs de thèse : Christophe Ferrari et Carlo Barbante

Date probable de soutenance de la thèse : Décembre 2008

Le Nouveau Chapitre de la Thèse par Raphaëlle Hennebelle

- **Présentation succincte**

Mon travail de thèse comportait plusieurs enjeux.

Tout d'abord au niveau scientifique le but principal était de mieux comprendre le rôle des bactéries et autres êtres microscopiques dans le cycle du mercure au Pole Nord.

L'enjeux sociétal d'un tel travail est donc de mieux comprendre la pollution par le mercure en Arctique et donc de mieux la maîtriser.

Au niveau technique cette thèse était un challenge car il a fallu utiliser des techniques d'analyses très pointues et peu communes.

D'un point de vue économique la compréhension d'un type de pollution peut avoir des répercussions non négligeables sur certains secteurs, comme dans notre cas sur ceux qui rejettent du mercure dans l'atmosphère.

Ma thèse porte sur les bactéries et autres microorganismes qui vivent dans la neige près du Pôle Nord. En effet, à cet endroit de la planète, il y a un polluant invisible qui se dépose sur la neige et qui est très toxique : c'est le mercure.

Selon sa forme, le mercure peut être très dangereux pour les animaux et les hommes car ingéré en petite quantité tous les jours il empoisonne à petit feu. Il existe sous forme de gaz ou de liquide, il est rejeté dans l'air lorsque l'on brûle des déchets ou par les pots d'échappement des voitures, il peut aussi être rejeté par des éruptions volcaniques ou par l'érosion de roches qui en contiennent. Une fois dans l'air, il reste très longtemps donc il peut être transporté partout dans l'atmosphère autour de la Terre.

Près du Pôle Nord, il y a des réactions particulières qui se produisent et qui vont faire que ce mercure de l'air va se déposer sur la neige. On sait depuis longtemps que les animaux vivant près du cercle polaire arctique sont empoisonnés par le mercure mais pour le moment personne ne sait comment ce poison arrive à rentrer en eux.

Mon travail a donc pour but de voir si les bactéries et autres animaux microscopiques sont capables de transformer le mercure qui se dépose sur la neige et donc si elles participent à empoisonner les animaux qui vivent près du Pôle Nord.

- **Ma thèse dans son contexte**

Je suis étudiante à l'Université Scientifique Joseph Fourier de Grenoble, au Laboratoire de Glaciologie et Géophysique de l'Environnement (L.G.G.E.) et je dépends de l'Ecole Doctorale Terre, Univers, Environnement.

Dans ce laboratoire je fais partie du thème « Chimie atmosphérique et interactions air-neige » qui rassemble une douzaine de chercheurs et une demi-douzaine de doctorants.

Dans cette équipe Chimie nous sommes un petit groupe à nous intéresser aux cycles des métaux lourds et en particulier au mercure.

Cette équipe est composée de :

- ✓ 2 Professeurs des Universités
- ✓ 1 Maître de conférence,
- ✓ 1 ATER
- ✓ 4 Thésards
- ✓ 1 Master 2
- ✓ 1 Master 1

Ma thèse est en cotutelle avec le laboratoire italien de Sciences Environnementales de l'Université Ca'Foscari de Venise.

Car je travaille avec des toutes petites quantités de mercure, si petites qu'il y a très peu de machine capable de les mesurer.

Ce laboratoire italien en possède une, c'est pourquoi je suis en cotutelle avec eux.

Au niveau international il y a plusieurs autres équipes qui travaillent sur ces problèmes d'empoisonnement au mercure au Pôle Nord. Il y a beaucoup de Canadiens et aussi des Norvégiens. Mais aucune de ces équipes ne travaille au même endroit que nous (Svalbard) ni sur les mêmes bactéries.

Cette thèse étant la première thèse en biologie dans cette équipe de chimistes, les compétences et les équipements ne sont pas disponibles au LGGE. Dans ce laboratoire j'ai tout de même mon bureau avec mon ordinateur, la salle blanche qui me sert à nettoyer mes flacons. J'ai accès à des imprimantes noir et blanc ou couleur, à des ressources bibliographiques, et je peux passer des commandes de matériel. Il y a également un service informatique, et un service technique pour m'aider si besoin est.

Pour tout ce qui est manipulation de bactéries, je travaille en collaboration avec le Laboratoire de Synthèse Et Etudes de Systèmes à Intérêt Biologique (SEESIB) de l'Université Blaise Pascal de Clermont Ferrand. Là bas je suis aidée par une ingénieure de recherche et une technicienne de laboratoire et l'on me fournit tout le matériel nécessaire pour mes manipulations (ou alors je l'amène de Grenoble).

Pour les analyses de mercure je travaille à l'université Ca'Foscari de Venise, où je suis aidée par un ingénieur de recherche.

Mon travail se situe donc entre les 3 laboratoires :

- ✓ LGGE de Grenoble
- ✓ SEESIB de Clermont Ferrand
- ✓ Laboratoire de Sciences Environnementales de Venise.

- **Moi dans ce contexte**

J'ai toujours aimé travailler dans des laboratoires, c'est pourquoi je m'étais initialement orientée vers des études de technicienne de laboratoire (DUT Génie Biologique à Lille).

Durant ces études j'ai découvert la microbiologie, matière qui m'a beaucoup plu et intéressée. De plus à la fin de ces études j'ai fais un premier stage dans un laboratoire de recherche (Laboratoire de Technologie des Substances Naturelles de Lille 1) et cela m'avait également plu.

J'ai ensuite continué mes études en DEUG de Biologie à Grenoble. A l'époque je n'envisageais pas encore la recherche mais plutôt l'enseignement, ayant été animatrice en centre de loisirs je visais la licence pour ensuite passer les concours de professeurs des écoles.

La licence biologie des organismes que j'ai suivie ensuite était plutôt orientée vers le fonctionnement de la nature et cela m'a passionné. J'ai donc finalement continué en Maîtrise (Biologie, Ecologie et Environnement)

En fin de Maîtrise j'ai fais un autre stage dans un laboratoire de recherche (Laboratoire d'Ecologie Alpine de Chambéry), ce qui m'a finalement donné envie de continuer dans cette voie.

Toujours passionnée par la nature et l'environnement j'ai finalement voulu m'intéresser à l'impact de la nature polluée sur l'homme, et j'ai donc terminé par un Master 2 Méthodes de Recherches en Environnement et Santé.

En Master 2 j'ai été très intéressée par la proposition de stage sur le mercure et les bactéries faites par le LGGE.

En effet elle combinait la microbiologie et l'étude d'un polluant dangereux pour la santé. J'ai donc effectué mon stage de Master2 sur ce sujet.

Durant ce stage nous avions eu de bons résultats et mon directeur disposait d'une bourse de thèse je me suis donc lancée dans l'aventure.

L'idée de voyager et de rencontrer des chercheurs de différents pays m'a également motivée.

A mon arrivée il a fallu écrire des demandes de financement (ANR) et comme mon équipe était composée de chimistes, j'ai dû aider à la rédaction de ces ANR en amenant mes compétences de biologiste.

J'ai donc participé à développer la place de la biologie au LGGE car les résultats encourageants de mon Master 2 puis du début de ma thèse, ont permis de recruter d'autres personnes sur cette thématique.

En tout 3 biologistes (2 thésards et 1 ATER) ont été recrutés suite aux résultats obtenus.

Depuis j'ai pas mal participé à définir les orientations de ma thèse.

- **Déroulement, gestion et coût de ma Thèse :**

- **moyens financiers**

Mon directeur de thèse avait eu une ACI jeune chercheurs juste avant mon arrivée, de plus en tant que membre de l'Institut Universitaire de France il avait également des crédits de ce côté là. Je disposais donc de moyens financiers pour commencer ces recherches. Nous avons ensuite écrit des ANR afin d'avancer dans cette voie.

- **antériorité de l'équipe dans la discipline**

Au LGGE, mon équipe travaillait sur le mercure dans l'atmosphère et son dépôt sur la neige. L'idée de s'intéresser aux bactéries de la neige était très novatrice et n'avait pas de précédent.

Au début de ma thèse cette équipe métaux était réduite. Elle se composait des 2 Professeurs des Universités, de 2 thésards et de 2 Master 2 (dont moi). J'étais la seule biologiste, donc finalement à Grenoble j'ai interagit avec mon directeur. A Clermont Ferrand j'ai travaillé avec des biologistes avec qui l'interaction était plus facile.

- **moyens techniques**

Le LGGE ne disposait pas des moyens techniques nécessaires à la réalisation d'expériences en microbiologie, j'ai donc dû travailler avec les 2 autres laboratoires : Clermont Ferrand pour la Microbiologie et Venise pour les analyses de mercure.

- **moyens logistiques**

Les risques d'un travail entre plusieurs laboratoires étaient beaucoup liés à la logistique, car il fallait préparer le matériel à Grenoble, puis l'envoyer à Clermont Ferrand, une fois l'expérience faite, il fallait finalement envoyer les échantillons congelés à Venise afin de les analyser.

- **sujet novateur**

Le côté très exploratoire de ma thèse était le principal facteur de risque mais c'était aussi son principal facteur de succès. Car bien que nous ayons eus des résultats en Master 2, nous ouvrions tout de même une thèse entière sur un sujet très peu étudié.

De plus la gestion de la thèse entre 3 laboratoires m'a obligé à travailler en aveugle, c'est à dire sans pouvoir toujours contrôler ce que je faisais, ce qui a compliqué beaucoup de points, mais cela m'a aussi permis d'apprendre à m'organiser et de réaliser qu'une collaboration entre plusieurs partenaires nécessitait en plus des qualités scientifiques, des qualités humaines et des qualités de logisticien.

- **Choix des partenaires nationaux et internationaux**

Le choix du laboratoire de Sciences Environnementales de Venise est basé sur leurs compétences reconnues en termes d'analyses de métaux traces dans des carottes antarctiques. En effet cette équipe travaille en collaboration étroite avec la notre sur les carottes de glaces et plusieurs étudiants ont été en cotutelle entre nos laboratoires c'est tout naturellement que ma thèse s'est inscrite dans cette continuité.

Un des étudiants du SEESIB de Clermont Ferrand, avait participé à une mission au Svalbard en 2004. Cette mission a permis l'isolement et l'identification de bactéries issues de la neige. De plus, de part leur travail sur les bactéries des nuages et sur la biodégradation de polluants dans les sols, les compétences du SEESIB en microbiologie sont reconnues.

C'est donc en toute logique que nous avons décidé de continuer à travailler avec eux sur ce thème, n'ayant pas de laboratoire de microbiologie à disposition à Grenoble.

Nous avons également collaborés avec le GEOTOP de l'Université du Québec à Montréal pour des analyses de mercure et méthylmercure. En effet, suite à une conférence (Mercury As a Global Pollutant, Madison, USA, Août 2006), j'ai rencontré une équipe québécoise qui travaillait sur des problématiques de contamination des poissons par le mercure. Experts en analyse du mercure dans les êtres vivants, j'ai monté avec eux une collaboration pour analyser des échantillons de biofilms arctiques en antarctiques.

Plus récemment, j'ai collaboré avec une équipe de l'université de Bordeaux pour des analyses de mercure dans des échantillons de plancton collectés au pôle nord.

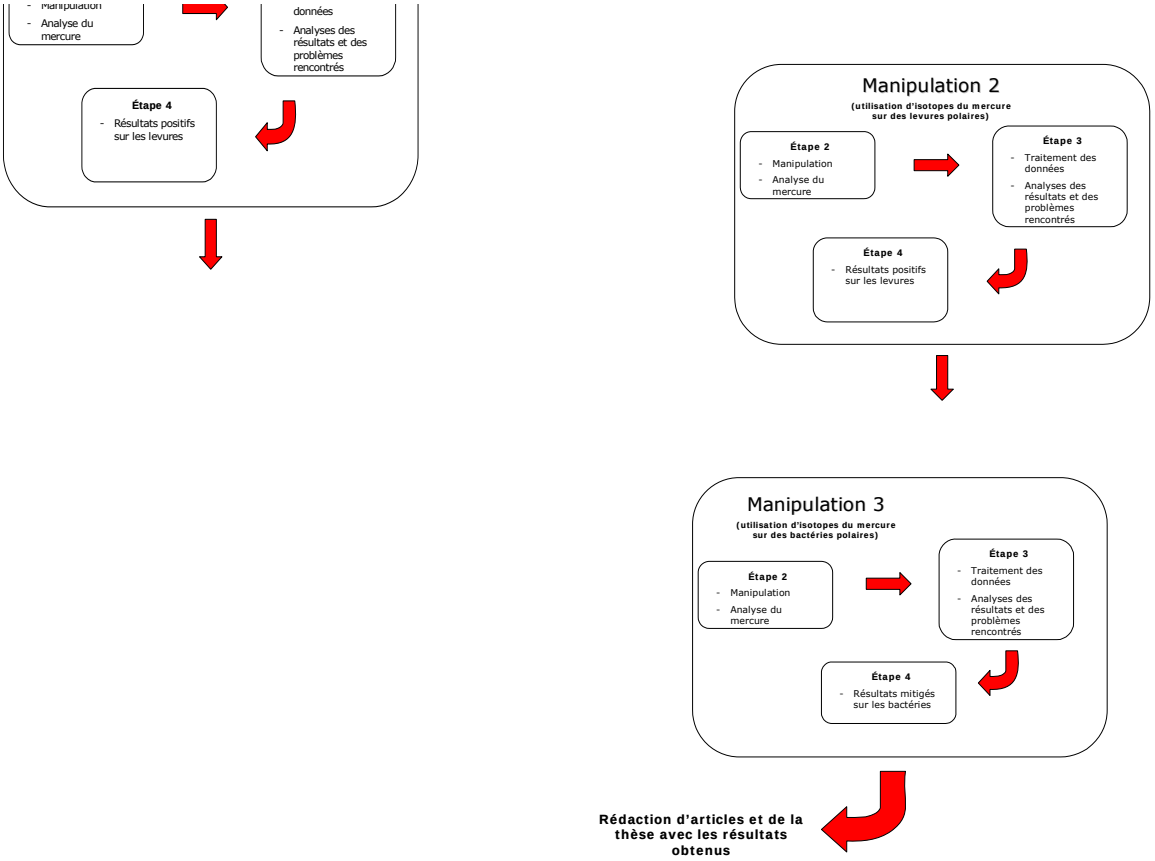
Pour financer mes voyages en Italie j'ai monté 2 dossiers de financement qui ont été acceptés et m'ont permis de financer mes déplacements :

- un premier programme financé par la région m'a été accordé : le programme Eurodoc qui représentait une somme de 2000 Euros pour le laboratoire et 2000 Euros pour moi

- un deuxième programme financé par l'Université Franco-Italienne : le programme Vinci (environ 2000 Euros pour le labo)

Pour les analyses de mercure et méthylmercure dans des échantillons de biofilms arctiques et antarctiques effectuées à Montréal j'ai posé un candidature au Centre de Coopération Inter-universitaire Franco-Québécois (Paris) et j'ai obtenu une bourse de 1000 euros qui m'a servie à financer une partie de mon voyage au Québec.

- **Conduite du projet**



Les différentes étapes de mon travail de thèse

Ma thèse avait pour objectif de comprendre comment les bactéries et les microorganismes de la neige se comportaient par rapport au mercure.

Ceci s'est déroulée en plusieurs étapes, au début j'ai eu un comité de thèse qui s'est réuni afin de s'assurer que je partirai dans la bonne direction, de plus mon directeur de thèse était assez présent pour m'aider au bon lancement de ce travail.

J'ai d'abord tiré un bilan de mon stage de Master2, puis élaboré une manipulation plus pointue (Manipulation 1). Une fois le protocole soumis à tous les partenaires et validés par eux, j'ai pu commencer à manipuler à Clermont Ferrand puis à aller analyser les quantités de mercure à Venise.

Les premiers résultats ont été plutôt encourageants, Mais nous avions tout de même des problèmes de contamination par du mercure « extérieur » car je travaille à basse concentration. Malgré cela les levures polaires donnaient des résultats intéressants.

Pour s'affranchir des problèmes de contamination j'ai donc décidé de refaire cette expérience sur les 3 levures que j'avais, mais en utilisant cette fois ci, du mercure et du méthylmercure isotopique (= mercure et méthylmercure marqué, Manipulation 2), en effet cela permettait de s'affranchir des problèmes de contamination et également d'étudier le comportement des levures avec du méthylmercure en plus du mercure.

J'ai donc refait cette expérience avec mes 2 mercures marqués, malheureusement la première fois elle n'a pas marché. J'ai recommencé et là les résultats ont été très intéressants : les levures polaires dépolluent le mercure et stockent le méthylmercure.

Suite à ces résultats j'ai voulu tester cette expérience sur des bactéries (Manipulation 3), ce que j'ai fait en mars 2008, les résultats sont encore en cours de traitement.

La plupart des décisions ont été prises avec mon directeur de thèse et l'avis des chercheurs concernés (de Clermont-Ferrand ou de Venise)

Parallèlement à tout cela, j'ai aussi effectué des mesures de mercure et méthylmercure sur des biofilms polaires (GEOTOP, UQAM, Montréal), ainsi que sur du plancton récolté en Arctique lors de la campagne polaire (Université de Bordeaux).

- Gestion des relations avec les partenaires scientifiques, socio-économiques, industriels, sous-traitants…

Les relations avec les différents partenaires n'ont pas toujours été faciles !

A Clermont Ferrand il m'a fallu un peu de temps pour me faire accepter, en effet la collaboration avait été montée par mon directeur de thèse et la chef du laboratoire de Clermont, or les chefs ne travaillent pas vraiment dans le laboratoire, les techniciens et ingénieurs avec qui j'ai eu a travailler par la suite m'ont vu débarquer de nulle part ! Il a donc fallu un peu de temps pour que chacun trouve ses marques, moi dans leur laboratoire et eux avec moi.

La plupart du temps je manipulais seule, mais il y avait souvent quelqu'un qui vérifiait plus ou moins que je savais me débrouiller.

C'était la même chose en Italie, la première fois que j'y suis allée, on m'a montré comment allumer la machine puis comment elle marchait grosso modo et ensuite je me suis débrouillée toute seule. Quand j'avais un problème je finissais toujours par trouver quelqu'un pour m'aider à le résoudre (technicien, étudiant, post doc etc.).

La plupart des problèmes étaient donc d'ordre humain, et à force d'être là j'ai fini par avoir ma place et par être considérée comme faisant partie du labo que ce soit à Clermont Ferrand ou à Venise.

- **Evaluation et prise en charge du coût de mon projet**

- **Ressources Humaines :**

Personnel	Temps en mois	Coût moyen mensuel + 40 % de charges patronales	Coût Total	Origine des Fonds
Doctorant	36	2 321,55	83 575,8	UJF
Directeur de Thèse	3,3 jours par mois pendant 36 mois	4 909,41	26 510,814	UJF
Ingénieur d'Etude (Clermont)	2 jours par semaines pendant 5 mois	3 741,39	9 353,475	UBP
Ingénieur d'Etude (Venise)	2 jours par semaine pendant 3 mois	3 741,39	5 612,085	UCF
Technicien TCN	1 jour par semaine pendant 5 mois	2 547,96	2 547,96	UBP
Encadrement Québec	0,5	3 492,79	1 746,395	UQAM
Stagiaire Master 1	2	500	1 000	UJF
		Total RH	**130 346,52**	**Euros**

- **Coûts des Manipulations :**

Détails		Coûts	Origine des Fonds
Campagne de terrain pour obtention d'échantillons	50 euros/jours pendant 60 jours + 2000 euros billets avion + 2792,59 euros pour rapatriement des échantillons	14 792,59	IPEV
Commandes de matériel pour réaliser les expériences	inclus tout le matériel qui a été commandé	5 881	66% CNRS, 34% UJF
Déplacements entre les 3 laboratoires	somme des coûts des moyens de transports, de l'hébergement sur place et des repas (- 6000 euros ont été couverts par Vinci+Eurodoc)	4 617,25	UJF
Coûts logistiques	coûts liés au déplacement du matériel et à l'envoi des échantillons congelés entre les laboratoires	6 151	65% CNRS, 35% UJF
Mesures sur l'ICP-MS	35 euros par analyses (600 analyses)+ prix des colonnes de chromatographie	21 700	UCF
	Total manipulations	**59 141,84**	**Euros**

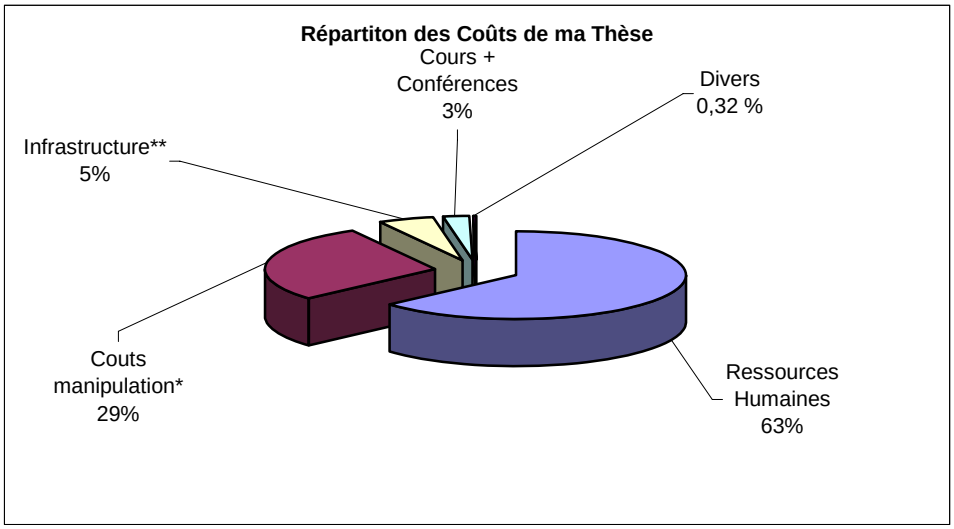
- **Autres Coûts :**

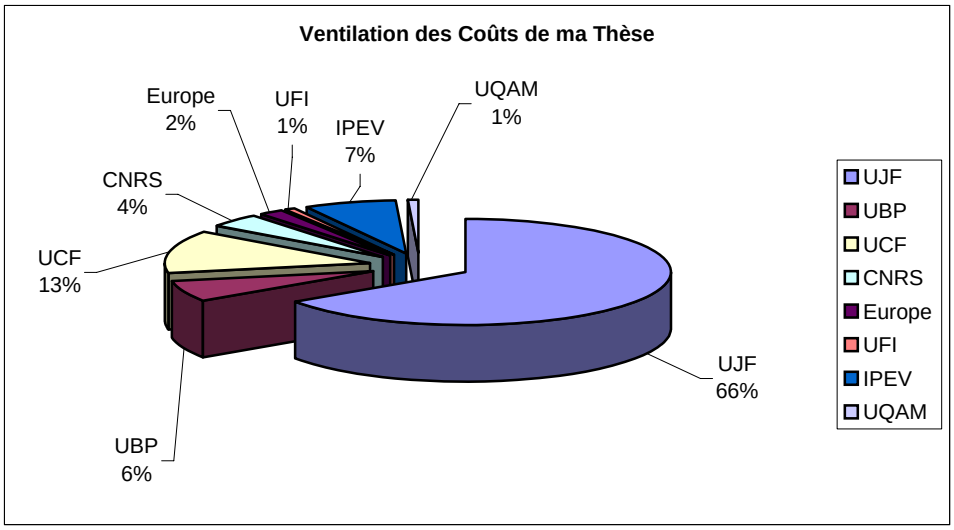
Conférences	Innsbruck + Madison + EGU + MOME	3 801	11% CNRS, 89 % UJF
Cours suivis	ERCA+ la recherche française : fonctionnement et recrutement + NCT (500 euros par formation environ)	1 500	UJF
	Total conférences et cours	**5 301**	**Euros**

Moyens mis à disposition	Coûts moyen pour un an	Coût moyen par personne / an	Coût Total	Origine des Fonds
Laboratoire de Clermont Ferrand	pour 5 mois	3 000	1 250	UBP
LGGE	pour 3 ans	3 000	9 000	UJF
Matériel Informatique	dur externe 1 écran et 1 clavier+endnote		555	UJF
Total Infrastructure			**10 805**	**Euros**

Divers	Impressions de poster et du manuscrit	600	UJF

TOTAL de la Thèse	**206 244,37 Euros**





Acronymes :

UJF : Université Joseph Fourier (Grenoble)

UBP : Université Blaise Pascal (Clermont Ferrand)

UCF : Université Ca'Foscari (Venise, Italie)

CNRS : Centre National de la Recherche Scientifique

Europe : Bourse Eurodoc

UFI : Université Franco-Italienne (Grenoble)

IPEV : Institut Paul Emile Victor

UQAM : Université du Québec à Montréal

- **Compétences, savoir-faire, qualités professionnelles et personnelles illustrées par des exemples**

Au niveau scientifique ma thèse m'a permis d'acquérir des nouvelles compétences en chimie environnementale et analytique et de renforcer mes compétences en microbiologie et d'aborder un problème scientifique dans sa globalité.

Cette expérience m'a beaucoup appris au niveau méthodologique et organisationnel. J'ai eu à gérer énormément de choses au niveau logistique (envoi de matériel, transport d'échantillons congelés, organisation de manip dans un planning serré, gestion d'une manip en « aveugle »)

Ayant beaucoup servi de lien entre mes 3 équipes de recherches j'ai appris à mieux communiquer, à animer des réunions entre les différents acteurs du projet et à aller à l'essentiel afin que tout le monde suive.

Il a aussi fallu que j'apprenne à visualiser ce qui était possible ou non et à le communiquer aux différents partenaires

J'ai également appris à gérer au mieux le temps que j'avais dans chaque laboratoire.

Tout cela m'a donc demandé de la rigueur et de l'organisation.

Les différents soucis que j'ai eu avec ma manipulation ainsi que son organisation entre 3 laboratoires, m'ont appris à m'adapter et à hiérarchiser mes priorités afin de faire des choix pertinents en très peu de temps.

Finalement gérer une expérience en faisant les commandes à Grenoble, la manipulation à Clermont et les analyses à Venise m'a obligé à savoir penser à tout voire plus !

Grâce à cette thèse j'ai aussi pu perfectionner mon anglais lors de mes séjours en Italie et lors des conférences auxquelles j'ai participé.

D'un point de vue relationnel, j'ai appris à m'intégrer dans différentes équipes, à travailler avec de nombreuses personnes différentes et à ménager les susceptibilités de chacun afin de faire avancer au mieux le projet.

J'ai également appris à monter des dossiers administratifs parfois rébarbatifs mais avec une grande satisfaction lorsqu'ils aboutissaient (aide pour dossiers ANR, différents dossiers de bourses comme Vinci et Eurodoc). J'ai aussi découvert les relations avec les fournisseurs de matériel scientifique en passant des commandes, et en dialoguant avec eux.

Durant cette thèse j'ai finalement pu développer des qualités en encadrement car j'ai effectué, en 1ere année de thèse, 68h de vacations à l'IUT de Chimie (encadrement de Travaux Pratiques de Chimie Organique).

J'ai également encadré un Stagiaire de Master 1 et pas mal aidé un stagiaire de Master 2.

- **Qualités personnelles : créativité, innovation, capacité d'analyse et de synthèse, aptitudes à l'encadrement et à l'animation, rigueur, adaptabilité…**

La thèse m'a permis de développer mes capacités d'adaptation et de communication, en effet au fil de son avancée, les informations entre les 3 équipes passaient de mieux en mieux et il est devenu de plus en plus facile pour moi de faire comprendre ce que je faisais et où j'en étais.

Au long de ces trois ans j'ai aussi beaucoup pris confiance en moi communiquer mes idées et points de vue scientifiques dans une réunion ne me pose plus de problème.

- **Construction d'un réseau professionnel personnel**

A la suite d'une conférence en Autriche j'ai développé une collaboration avec des chercheurs espagnols qui nous ont fourni des échantillons de biofilms polaires, de la même façon lorsque je suis allée à Madison j'ai rencontré des chercheurs de l'UQAM de Montréal chez qui je suis venue faire des analyses l'année suivante.

J'ai toujours eu d'excellentes relation avec mes 2 laboratoires d'accueil (Clermont et Venise). Et lors de la campagne polaire, j'ai rencontré des chercheurs qui travaillaient sur le plancton et j'ai monté une collaboration avec eux.

- **Transférabilité de ces compétences en dehors de votre domaine de recherche**

Dans cette partie je vais présenter différents métiers pour lesquels je pourrai convenir.

J'ai volontairement choisi 3 métiers différents les uns des autres car il me paraissait évident qu'après une thèse les postes d'ingénieurs de recherche ou d'ingénieur d'étude dans le privé étaient tout à fait atteignables.

Pour chaque métier suggéré j'indiquerai les compétences que je possède et celles qu'il me faudra acquérir.

Le pourcentage final de compatibilité est calculé en comptant le nombre de compétences que j'ai sur le total nécessaire.

Pour être : Cadre technique de l'environnement

Je saurai :

- Mettre en place des méthodes et des moyens de protection afin de réduire la fréquence des risques, mais aussi d'en diminuer l'ampleur et les conséquences.
- Surveiller les installations (station de détoxication, centre d'enfouissement, station d'épuration...) pouvant provoquer des nuisances sur l'environnement
- Faire respecter les règles de protection de l'environnement
- Effectuer des prélèvements sur des sites suspects
- Analyser, examiner et formaliser les résultats des prélèvements réalisés
- Maîtriser une ou plusieurs langues étrangères, notamment l'anglais ou l'allemand
- Analyser et synthétiser des informations techniques et organisationnelles

Il me faudra me former sur :

- Proposer l'exécution de travaux permettant de diminuer les nuisances du site
- Connaître et utiliser les méthodes et matériels informatiques (logiciels, matériels, microprocesseurs...).
- Posséder les techniques de traitement statistique de données.
- Négocier avec des interlocuteurs variés et prendre en compte leur avis.
- Anticiper les risques et les dysfonctionnements

➔ compatibilité métier : 7 / 12 soit 58.3 %

Pour être : Journaliste scientifique

Je saurai :

- Choisir un événement, un fait ou un thème (informations événementielles, sujets socioculturels, scientifiques ou techniques...) devant intéresser son public. ·
- Chercher et recueillir l'information et la vérifier. ·
- Traduire ou faire traduire l'événement, le fait ou le thème dans un langage médiatique pour produire une information adaptée au support et au public. ·
- Pratiquer une ou plusieurs langues étrangères·
- M'intéresser à l'environnement et savoir l'observer avec un regard sélectif. ·
- Faire abstraction de ses propres représentations et idées pour transmettre l'information avec objectivité.

Il me faudra me former sur :

- Pratiquer la publication assistée par ordinateur (PAO)
- Faire face à des situations pouvant comporter des risques physiques.
- Respecter des règles déontologiques.
- S'adapter aux impératifs du traitement de l'information
- Posséder un "carnet d'adresses"

➔ compatibilité métier : 6 / 11 soit 54.5 %

Pour être : Animatrice spécialiste d'activités culturelles et techniques

Je saurai :

- Organiser un programme d'activités prenant en compte une progression pédagogique
- Enseigner les techniques de base de l'activité
- Expliquer le fonctionnement et le maniement des matériels et des équipements à utiliser
- Animer les activités
- Faire acquérir et développer les habiletés et les comportements propres à la technique
- Assurer les tâches matérielles de préparation et de logistique

- Evaluer les acquis et les résultats.
- Travailler en concertation et en complémentarité avec une équipe

Il me faudra me former sur :

- Posséder des connaissances en psychopédagogie appliquée
- Posséder le Brevet national de secourisme (BNS).
- Posséder un Brevet de surveillance de baignade
- Développer des relations accueillantes et dynamiques avec le public concerné
- Etre imaginatif et créatif
- Actualiser ses connaissances
- Exercer une autorité.

➔ compatibilité métier : 8 / 15 soit 53.3 %

- **Résultats, impact de la thèse**

Mon travail de thèse a permis d'ouvrir tout un pan de recherche encore jamais exploré au Laboratoire de Glaciologie.

La biologie dans ce laboratoire débute et mes résultats n'y sont pas pour rien. Au niveau de ma propre équipe, mes travaux ont permis le recrutement de 2 thésards et d'une ATER sur des sujets ayant trait à la biologie. En ce qui concerne nos partenaires ils ont pu profiter du rayonnement de notre laboratoire et avoir plus de financements pour leurs projets.

En montrant que les micro-organismes de la neige pouvaient réagir avec un polluant comme le mercure j'ai prouvé que l'étude de l'environnement n'était pas qu'une simple question de chimie.

J'espère que mon travail aura permis de développer une nouvelle vision des sciences environnementales intégrant tous les paramètres, y compris le vivant.

Finalement au niveau de la société je pense que j'ai participé à faire prendre conscience de la dangerosité de la contamination des pôles par le mercure (intervention à la Télévision Québécoise et sur Euronews), ce polluant est connu depuis longtemps mais l'opinion publique ignorait jusqu'ici les risques et problèmes encourus, cela commence à changer tout doucement.

En tout cas, au vue du chemin parcouru plus rien ne me fait peur !

Ma vision de la recherche a beaucoup changé en trois ans et pour le moment j'en sors plutôt déçue (mais il paraît que c'est normal en fin de thèse !). Bien que j'ai fait des choses très intéressantes et que j'ai pu voyager, cette thèse m'a aussi montré un milieu très élitiste, pas forcément très humain et pas toujours à la hauteur des espérances que l'on y place.

C'est pourquoi je me pose des questions sur mon avenir dans ce milieu. dans tous les cas si je dois rester dans la recherche ce sera à des postes d'ingénieur de recherche ou de chercheur CNRS mais pas aux postes de maître de conférence.

En effet, et bien que l'enseignement soit quelque chose qui m'intéresse, les postes d'enseignants chercheurs ont, à mes yeux, un service d'enseignement trop important qui, si l'on veux le faire correctement, ne laisse pas beaucoup de temps pour la recherche ou alors très peu pour la vie privée.

De plus les salaires dans la fonction publique ne compensent pas l'investissement en temps que nécessite un tel métier.

C'est pourquoi je pense que travailler dans la recherche privée me satisferais plus. D'autant que la transférabilité de mes connaissances et de mes savoir-faire paraît tout à fait possible, en espérant que les entreprises reconnaissent enfin la valeur d'un doctorat.

De plus toute la partie encadrement de stagiaires m'a plue, je compte refaire de l'animation après ma thèse pour voir si j'ai toujours un bon contact avec les enfants et je n'exclus pas la possibilité de passer les concours de professeur des écoles.

Sinon, je me sens prête à faire des tas de choses, j'aime beaucoup écrire et tout ce qui touche au journalisme, scientifique ou non, pourrait également me convenir.

Dans tous les cas cette expérience aura été très enrichissante, au fil de son avancée cette thèse m'a fait prendre confiance en moi et m'a permis de me rendre compte que j'étais capable de faire beaucoup de choses, de travailler avec de nombreuses personnes différentes, y compris dans une autre langue et dans un autre pays ainsi que de régler les problèmes au fur et à mesure.

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Speciation analysis of mercury in seawater from the lagoon of Venice by on-line pre-concentration HPLC–ICP-MS

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ABSTRACT

A method based on the coupling of HPLC with ICP-MS with an on-line pre-concentration micro-column has been developed for the analysis of inorganic and methyl mercury in the dissolved phase of natural waters. This method allows the rapid pre-concentration and matrix removal of interferences in complex matrices such as seawater with minimal sampling handling. Detection limits of 0.07 ng L^{-1} for inorganic mercury and 0.02 ng L^{-1} for methyl mercury have been achieved allowing the determination of inorganic mercury and methyl mercury in filtered seawater from the Venice lagoon. Good accuracy and reproducibility was demonstrated by the repeat analysis of the certified reference material BCR-579 coastal seawater. The developed HPLC separation was shown to be also suitable for the determination of methyl mercury in extracts of the particulate phase.

1. Introduction

The most common methods currently in use for the speciation analysis of mercury species are chromatography typically Gas Chromatography (GC) or High Performance Liquid Chromatography (HPLC) coupled to an elemental specific detector such as inductively coupled plasma-mass spectrometry (ICP-MS) [1]. GC coupled with ICP-MS currently has some of the lowest reported detection limits [2] for mercury species with detection limits of 0.027 pg g^{-1} for methyl mercury (CH_3Hg) and 0.27 pg g^{-1} for inorganic mercury (Hg^{2+}) with solid phase microextraction (SPME) pre-concentration. Other detection methods such as atomic fluorescence spectroscopy with solid phase extraction [3] can reach detection limits as low as 0.01 ng L^{-1} for CH_3Hg and is suitable for the analysis of mercury species in ocean water [4]. However, the drawback of GC is that the species have to be rendered volatile and this requires a derivatisation step first with either Grignard reagents or more recently tetraalkyborate compounds [5] which can be time consuming and can sometimes result in species transformations [6], because of this another method for comparison is desirable.

HPLC on the other hand requires no derivatisation step, as the species do not need to be volatile before injection

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[7], simplifying the sample preparation considerably. However, to reach the detection limits required for environmental analysis, a pre-concentration step is necessary, the various pre-concentration methods used have been reviewed [8] and include on-line [7], and off-line [9] pre-concentration on various materials including C-18 micro-columns [10,11] and sulfhydryl cotton [12]. However, to successfully separate mercury species by HPLC, ion pairing agents such as L-cysteine [13,14] are required, which when coupled with vapour generation and ICP-MS gives detection limits of between 0.03 and 0.11 ng mL^{-1}. HPLC–ICP-MS with off-line pre-concentration [15] reached detection limits of 5.2 ng L^{-1} for Hg^{2+} and 5.6 ng L^{-1} for CH_3Hg, recently microbore HPLC–ICP-MS has been used for the speciation analysis of mercury [16], the use of a 1.0 mm i.d. analytical column operating at a flow rate of 70 μL min^{-1} significantly reduced the amount of solvent reaching the plasma leading to interesting sensitivity gains. This approach achieved detection limits of 11 ng L^{-1} for Hg^{2+} and 23 ng L^{-1} for CH_3Hg with no pre-concentration, however the large dead volume of the ICP-MS sample introduction system prevented the authors from fully exploiting the sensitivity that microbore HPLC should bring.

In this work a mid-bore (2.1 mm i.d.) HPLC column has been used, as the flow rates for these columns are most suitable for coupling with the low flow (<500 μL min^{-1}) higher sensitivity concentric nebulisers now available on the market. The reduced internal diameter means lower flow rates can be used, meaning that less solvent is introduced into the plasma increasing mass sensitivity, the mid-bore column geometry has the additional advantage of suffering less from dead volume effects, when compared to microbore HPLC. The use of L-cysteine and 2-mercaptoethanol in the mobile phase means that organic solvents and the problems related with them are avoided.

The replacement of the sample injection loop with a micro-column meant that large volumes (up to 5 mL) could be injected onto the pre-concentration column then eluted onto the analytical column, as the direct injection of 5 mL of sample onto the column could compromise the chromatographic resolution. This approach also allowed rapid on-line sample pre-concentration and matrix removal with minimal sample handling by the analyst for matrices as complex as seawater. Injection of the entire pre-concentrated sample instead of an aliquot as is the case for off-line pre-concentration resulted in low detection limits with minimal matrix effects while avoiding complex sample handling steps such as derivatisation.

The method was applied to seawater collected from the lagoon that surrounds the city of Venice. Monitoring of mercury in and around the city of Venice is important as it is World Heritage Site located uniquely in a coastal lagoon that from the 1950s through to the late 1980s was heavily contaminated with mercury by chlor-alkali process discharges from the nearby Marghera chemical works [17]. As fishing is still an important economic activity in this body of water, careful monitoring of the water quality of this delicate ecosystem is required, and the number of samples necessary for this means that rapid, and sensitive methods for monitoring important pollutants are required.

Table 1 – HPLC–ICP-MS operating conditions

Agilent 7500 is ICP-QMS	
Forward power	1450 W
Plasma gas flow	15 L min^{-1}
Auxiliary gas flow	1 L min^{-1}
Carrier gas flow	1.09 L min^{-1}
Sample depth	5 mm
Monitoring masses	*m/z* 184, 202 (1 point per peak)
Acquisition mode	Time resolved analysis
Integration time per mass	0.5 s
Spray chamber temperature	2 °C
Agilent 1100 series HPLC	
Column	100 × 2.1 mm Alltima HP C-18 3 μm particle size
Flow rate	0.2 mL min^{-1}
Injection volume	0.1–5 mL

2. Experimental

2.1. Instrumentation

The ICP-QMS used in this work was an Agilent 7500is (Agilent Technologies, Yokogawa Analytical Systems, Tokyo, Japan) fitted with a standard quartz spray chamber and a PolyPro-ST concentric nebuliser (Elemental Scientific Inc. Omaha, USA). This was coupled to an Agilent 1100 series HPLC pump (Agilent, Waldbronn, Germany) fitted with a manual injection valve (9125, Rheodyne, CA, USA) with a 100 μL (PEEK) sample loop (Alltech, Deerfield IL, USA), or an Opti-lynx™ 100 μL micro-column filled with a C-18 silica based packing material (Alltech, Deerfield IL, USA) instead of a sample loop. The mercury species were separated isocratically on a 100 × 2.1 mm Alltima HP C-18 3 μm column (Alltech, Deerfield IL, USA) at a flow rate of 0.2 mL min^{-1}, with a mobile phase of 0.5% L-cysteine (m/v) and 0.05% 2-mercaptoethanol (v/v) dissolved in ultra-pure water. The instrumental conditions are summarised in Table 1. The masses monitored were *m/z* 202, the most abundant mercury isotope and 184, a tungsten isotope to check for interference peaks from the formation of $^{184}W\ ^{18}O$.

2.2. Standards, reagents and materials

Mercury (II) chloride, methylmercury (II) chloride and 2-mercaptoethanol were purchased from Sigma–Aldrich (Milan, Italy) and the L-cysteine was purchased from VWR International (Milan, Italy). Stock standard solutions of approximately 1000 mg L^{-1} (as mercury) mercury chloride and CH_3Hg chloride were prepared by weight from the respective salts. Mercury chloride was dissolved in 1% (v/v) hydrochloric acid (Suprapur grade, Merck, Darmstadt, Germany) in a 100 mL amber glass bottle (Schott, Mainz, Germany); CH_3HgCl was dissolved in 10 mL of methanol (gradient UpS grade, Romil, Cambridge, UK) in a 100 mL amber glass bottle and made up to volume with 1% (v/v) hydrochloric acid, both solutions were stored refrigerated in the dark until required [18]. Working standards were made by serially diluting the stock standards with ultra-pure water in acid washed amber glass bottles (Schott, Duran, Mainz, Germany), samples were stored and

diluted in acid washed amber glass vials with PTFE liners (Supelco, Bellefonte, PA, USA). The water (18.2 MΩ) was generated by a Pure Lab Ultra water system (Elga Lab Water, High Wycombe, UK). Syringe filters when used were 0.45 μm cellulose acetate of 17 mm diameter (Alltech, Deerfield, IL, USA). The 0.2 μm cellulose acetate filters used for filtering the sea water samples were obtained from Sartorius (Germany), the accuracy and reproducibility of the method was checked by repeat analysis of the certified reference material BCR 579 coastal seawater certified for total mercury (IRMM, Geel, Belgium).

3. Results and discussion

3.1. Optimisation of the chromatographic separation

To maintain maximum sensitivity for the detector (in this case ICP-MS) it was decided to avoid methods using organic solvents. Of these the use of L-cysteine as an ion pairing agent seemed the most promising [13,19]. Fig. 1a shows a chromatogram of a mixed standard of 1 $\mu g L^{-1}$ of inorganic mercury and 1 $\mu g L^{-1}$ of CH_3Hg separated with a mobile phase of 0.5% L-cysteine at a flow rate of 0.2 mL min^{-1}, Fig. 1b shows the same standard separated with the same column and flow rate but with the addition of 0.05% (v/v) 2-mercaptoethanol to the mobile phase. It can be seen from Fig. 1b that the addition of this reagent has little effect on the area of the inorganic mercury peak, but has caused an increase in the peak height of the CH_3Hg peak, and a sharpening of both analytical peaks. The effect of further increases in the amount of 2-mercaptoethanol in the mobile phase can be seen in Fig. 2, this clearly shows that the addition of 2-mercaptoethanol increases the retention time for both analytes, but any increase above 0.05% (v/v) results in a significant loss in chromatographic resolution.

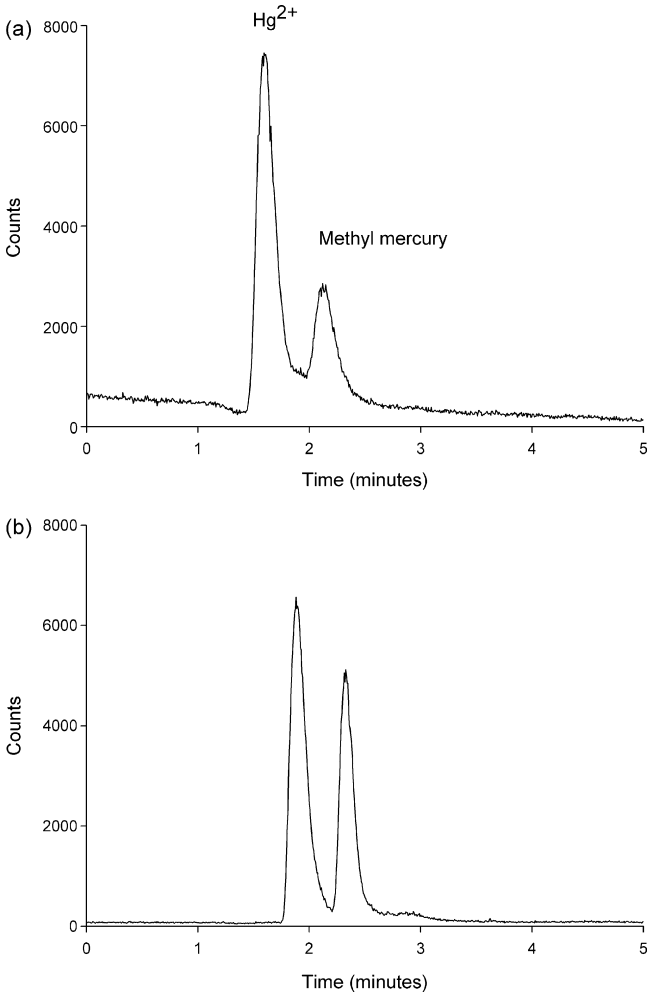

Fig. 1 – (a) The separation of a 1 $\mu g L^{-1}$ (100 μL injection) mixed inorganic mercury and CH_3Hg standard on a 100 × 2.1 mm Alltima HP C-18 3 μm HPLC column at a flow rate of 0.2 mL min^{-1} with a mobile phase of 0.5% (v/v) L-cysteine. (b) The separation of a 1 $\mu g L^{-1}$ (100 μL injection) mixed inorganic mercury and CH_3Hg standard on a 100 × 2.1 mm Alltima HP C-18 3 μm HPLC column at a flow rate of 0.2 mL min^{-1} with a mobile phase of 0.5% (m/v) L-cysteine and 0.05% (v/v) 2-mercaptoethanol.

3.2. Optimisation of the pre-concentration technique

To improve the sensitivity in order to detect mercury species at environmental levels, it was decided to include a pre-concentration technique. Aizpùn et al. [10] reported the use of a C-18 column modified with 2-mercaptoethanol to pre-concentrate the mercury species off-line, we decided to modify this method to an on-line method so that the entire pre-concentrated volume would be injected onto the column. This was achieved by replacing the 100 μL sample loop with a pre-concentration micro-column. The micro-column in this case is an Opti-Lynx™ trap cartridge with a bed volume of 100 μL, with an internal diameter of 4.6 mm and a length of 5.0 mm packed with a C-18 stationary phase. The sample is manually loaded onto the column using a standard glass HPLC syringe via the sample injection port, with the valve in the load position, sample elution is achieved by switching the valve to inject and the HPLC mobile phase elutes the analytes from the micro-column and transports them to the analytical column. Fig. 3 shows a chromatogram of a 100 μL injection of a 100 ng L^{-1} per species (as mercury) mixed standard of inorganic mercury and CH_3Hg prepared in 1% (v/v) HCl, and the same standard after the injection of a 1 mL aliquot onto the pre-concentration column before chromatographic separation. The peak areas for inorganic mercury and CH_3Hg after pre-concentration are increased by 10 and 6 times, respectively, which corresponds to the increased volume injected for inorganic mercury, but CH_3Hg appears to be pre-concentrated but with a roughly 50–60% efficiency.

Improvement of the pre-concentration of CH_3Hg was investigated by loading the pre-concentration column with higher concentrations of the individual reagents present in the mobile phase, this was done by injecting more concentrated solutions of 2-mercaptoethanol or L-cysteine onto the pre-concentration column to increase the ion pairing capacity. The effect of adding either 2-mercaptoethanol or L-cysteine to the standards was tried to increase the concentration of thiol–mercury complexes in solution. The results are summarised in Tables 2a and 2b as percent (%) recover-

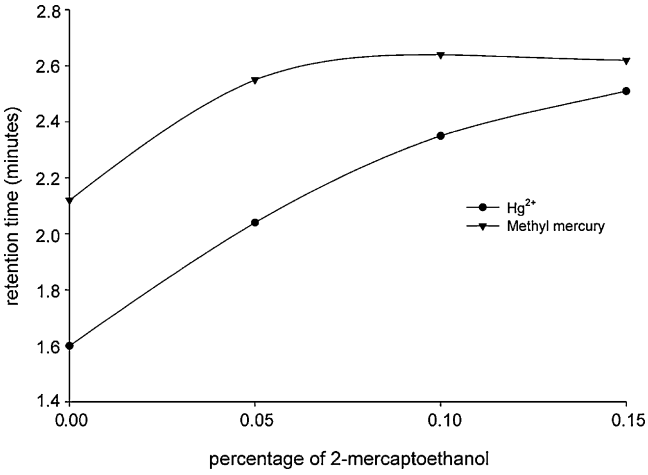

Fig. 2 – The effect of increasing the concentration of 2-mercaptoethanol in the mobile phase on the separation of inorganic and CH_3Hg by HPLC using a 100 × 2.1 mm Alltima HP C-18 3 μm HPLC column at a flow rate of 0.2 mL min^{-1} with a mobile phase of 0.5% (m/v) L-cysteine and increasing concentrations of 2-mercaptoethanol.

ies compared to the integration results of standards injected under the same standard conditions as listed above (the pre-concentration column preconditioned with the mobile phase and standards prepared in 1% (v/v) HCl). The percent recovery is calculated as the (integration results new conditions/integration results with standard conditions) × 100.

The results in Table 2a shows that loading the column with more reagents such as L-cysteine or 2-mercaptoethanol has a detrimental effect on the pre-concentration capability of the column, and that washing the column with 1 mL of ultra-pure water before use to remove them also had a negative effect demonstrating that the compounds present in the mobile phase play an important part in the pre-concentration mechanism.

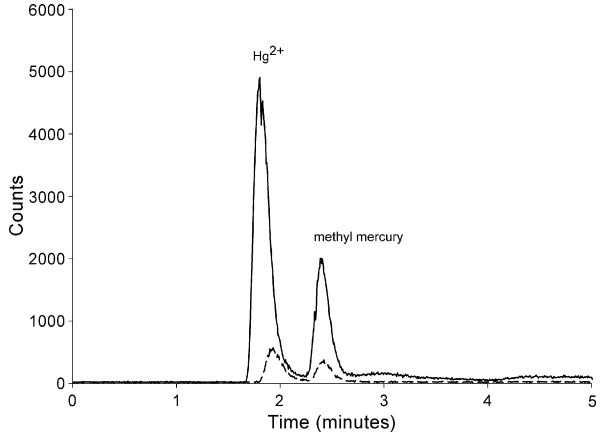

Fig. 3 – A chromatogram of a 100 μL injection of a 100 ng L^{-1} per species (as mercury) mixed standard of inorganic mercury and CH_3Hg prepared in 1% (v/v) HCl (small peaks, dashed line), and the same standard after the injection of a 1 mL aliquot onto the pre-concentration column before chromatographic separation (large peaks, solid line).

Table 2a – Recovery (%) of the mercury species with different column pre-treatments compared to their pre-concentration with column pre-conditioning with the HPLC mobile phase (0.5% (m/v) L-cysteine and 0.05% (v/v) 2-mercaptoethanol) with the species standards prepared in 1% (v/v) HCl

Pre-concentration column pre-treatment	Percent recovery (%)	
	Hg^{2+}	CH_3Hg
Injection of 1 mL of 0.2% (v/v) 2-mercaptoethanol	81	38
Injection of 1 mL of 1% (m/v) L-cysteine	16	11
Injection of 1 mL ultra-pure water	89	21

Table 2b – Recovery (%) of the mercury species in different standard matrices on a C-18 micro-column pre-conditioned with the mobile phase

Standard matrix	Percent recovery (%)	
	Hg^{2+}	CH_3Hg
Standard prepared in 0.05% (v/v) 2-mercaptoethanol	46	21
Standard prepared in 0.5% (m/v) L-cysteine	45	72
Standard prepared in 0.2% (v/v) HCl	136	114
Standard prepared in water	155	348

Table 2b shows that making the standards in 2-mercaptoethanol or L-cysteine showed no improvement, with a net reduction in analyte recovery demonstrating that inorganic mercury and CH_3Hg bind to the ion pairing reagents by forming on column complexes with L-cysteine and 2-mercaptoethanol immobilized on the stationary phase, rather than forming complexes in solution that then have an affinity for the stationary phase. The results in dilute hydrochloric acid and water show the only pre-concentration improvement, showing that mercury and above all CH_3Hg binds to thiols at neutral or a slightly acidic pH. This is in agreement with Percy et al. [14] who reported that at a pH between 5.0 and 8.0 cysteine is present as a zwitterion with the carboxyl group deprotonated (pK_a 1.95), the amino group protonated (pK_a 9.05) and the sulfhydryl group protonated. Our results are further supported by the findings of Rabenstein and Fairhurst [20] who reported that the sulfhydryl group binds CH_3Hg most strongly with a formation constant for CH_3Hg cysteine complexes of 5.0×10^{15} but that at pH < 2 this complex disassociates due to competition of protons for the sulfhydryl group.

In Fig. 4 the effect of sample volume (injection volume) on the pre-concentration of a mixed 10 ng L^{-1} Hg^{2+} (closed circle symbols) and CH_3Hg standard (open circle symbols) in ultra-pure water is reported, showing a linearity up to a pre-concentration volume of 20 mL for methyl mercury when the standards are made in ultra-pure water, but the Hg^{2+} profile is curved demonstrating that the break through volume maybe close to 20 mL. It proved to be impractical to inject larger volumes accurately with a 1 mL syringe making it difficult to obtain a precise determination of the break through volume,

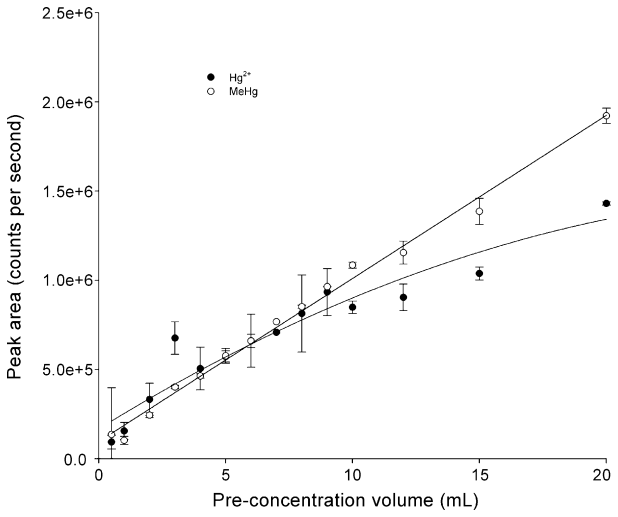

Fig. 4 – The effect of sample volume (injection volume) on the pre-concentration of a mixed 10 ng L^{-1} Hg^{2+} (closed circle symbols) and CH_3Hg standard (open circle symbols) in ultra-pure water ($n = 3$, error bars of 2S.D.).

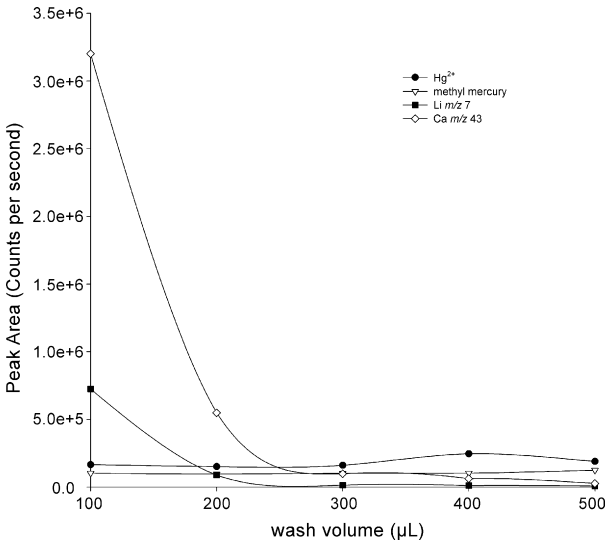

Fig. 5 – The effect of column wash volume on the recovery of mercury species (Hg^{2+} closed circles, CH_3Hg inverted open triangle symbols) from the pre-concentration column and elimination of the seawater matrix (Li filled square symbols, Ca open diamond symbols).

due to small errors in the amount of sample taken up each time and human error resulting in smaller volumes injected than expected. Using larger syringes made it more difficult to push the liquid onto the column, the largest practical syringe volume was found to be 5 mL, the back pressure generated by the pre-concentration column when using syringes above this volume caused the removable needles being used to lose liquid causing inaccuracies in the amount injected. The back pressure generated also proved to be too high for the use of a peristaltic pump to load the column with the low pressure fittings available in the laboratory.

The pre-concentration volume possible for real samples was then investigated by spiking a filtered seawater sample (filtered with a 0.2 μm membrane filter) with a mixed 10 ng L^{-1} Hg^{2+} and CH_3Hg standard and injecting it undiluted onto the pre-concentration column. A large characteristic sodium emission was observed in the bullet region of the plasma when the column was not washed after injection of a 1 mL sample of seawater, due to elution of the seawater matrix. Different wash volumes with ultra-pure water between 100 and 500 μL were investigated; removal of the seawater matrix was monitored by measuring Ca at *m*/z 43 and Li at *m*/z 7, and recovery of the mercury species by measuring the peak areas of the repeated 1 mL injections of the standard in seawater. The results are reported in Fig. 5, these show that Li is eliminated after a wash volume of 300 μL and 500 μL is required to return the Ca signal to baseline levels. Observing the plasma showed the sodium emission disappeared after washing with 200 μL, but the levels of Na present saturated the detector at wash volumes below 300 μL, making it impractical to use *m*/z 23 for monitoring of the washing process. The mercury recoveries after washing were unchanged so 500 μL was adopted as the washing volume.

To maintain the low blank levels necessary and avoid carry over between samples, the sample syringe was washed three times between samples or standards, the first wash was with 1% (v/v) HCl and the last 2 washes were with ultra-pure water in 2 different sample bottles so a cleanliness gradient was effectively achieved for the syringe washing solutions. The first wash solution instead of being discharged to waste was injected into the injection valve while in the inject position, to clean the injection port and internal flow lines that were not being effectively cleaned by the mobile phase. For the column washing for seawater samples, a separate 500 μL cleaned glass syringe was used to exclusively inject the column washing solution of ultra-pure water taken from the third wash solution bottle to avoid adding mercury to that already pre-concentrated on the column.

Having demonstrated that the washing protocols were effective, it was attempted to find the breakthrough volumes for Hg^{2+} and CH_3Hg in undiluted seawater, the results can be seen in Fig. 6, demonstrating that the break through volume for Hg^{2+} maybe close to 20 mL, but the curve for CH_3Hg is linear up to 20 mL demonstrating that much higher volumes can be pre-concentrated than those that can be injected using a syringe, indicating that these columns may be suitable for use to pre-concentrate the mercury species present off-line.

3.3. *Calibration and analytical figures of merit*

As mercury is only stable for short time periods when it is in unacidified solutions [21,18], the sample and standard handling protocol of Planchon et al. [22] was applied with the modification that all the standards were made in amber glass bottles. Mixed analytical standards between 0 and 100 ng L^{-1} were made fresh in ultra-pure water from acidified mother solutions (the concentrations of these were periodically checked against a certified mercury standard), samples were stored at −20 °C before analysis and were analysed immediately after defrosting without any acidification.

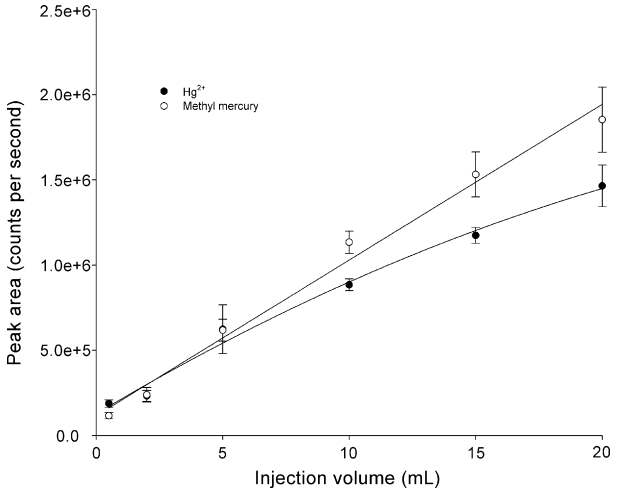

Fig. 6 – The effect of sample volume (injection volume) on the pre-concentration of a mixed 10 ng L^{-1} Hg^{2+} (closed circle symbols) and CH_3Hg standard (open circle symbols) in undiluted filtered seawater ($n = 3$, error bars of 2S.D.).

The figures of merit for injections of 20 mL of standard are reported in Table 3a. The reproducibility tests for the standard injections were carried out on new freshly made standards, as under these conditions the standards are only stable for an hour at most. From Table 3a it can be seen that the external calibration is linear over the calibration range and that the detection limit for CH_3Hg is better than that for inorganic mercury. The explanation for this is that the detection limit for inorganic mercury is blank limited as there is inorganic mercury present in all the reagents used during analysis. To test the accuracy of the external calibration a standard additions calibration for Hg^{2+} and CH_3Hg in a filtered seawater sample was carried out using the same calibration range and injection volumes, the results are reported in Table 3b, a separate spike of 10 ng $L^{-1}$was made on the same sample and repeatedly injected to find the spike recovery compared to the external calibration curve. In addition to this the accuracy and reproducibility of the method was checked by repeat analysis of the certified reference material BCR 579 coastal seawater certified for total mercury. Analysis of this material revealed that the mercury was wholly present as inorganic mercury, so an aliquot was spiked with 2 ng L^{-1} of CH_3Hg to check the spike recovery for this analyte. The results can be seen in Table 4, and show that with an external calibration method

Table 3a – Analytical figures of merit for the HPLC–ICP-MS with the micro-column pre-concentration method over a calibration range of 0–100 ng L^{-1} in ultra-pure water

Figures of merit in pure water	Hg^{2+}	CH_3Hg
Regression slope of linear range 0–100 ng L^{-1} (cps/ng L^{-1})	56,241	102,424
Linear regression coefficient (r^2)	0.9993	0.9994
Precision of peak area, 10 ng L^{-1} (%R.S.D.) ($n = 3$)	13.1	28.6
Limit of detection (3 × S.D. of concentration for a 0.5 ng L^{-1} standard) ($n = 5$)	0.07	0.02
Blank equivalent concentration (ng L^{-1})	1.18	0.21

Table 3b – Analytical figures of merit for the HPLC–ICP-MS with the micro-column pre-concentration method over a calibration range of 0–100 ng L^{-1} in filtered unacidified seawater

Figures of merit in seawater	Hg^{2+}	CH_3Hg
Regression slope of linear range 0–100 ng L^{-1} (cps/ng L^{-1})	64,600	90,782
Linear regression coefficient (r^2)	0.9968	0.9986
Precision of peak area, 10 ng L^{-1} (%R.S.D.) ($n = 3$)	8.9	5.5
Limit of detection (3 × S.D. of concentration for a 0.5 ng L^{-1} standard) ($n = 5$)	0.12	0.03
Blank equivalent concentration (ng L^{-1})	0.78	0.07

Table 4 – Spike recovery for an aliquot of filtered unacidified seawater spiked at 10 ng L^{-1} with Hg^{2+} and CH_3Hg versus an external calibration with standards made in ultra-pure water, and the accuracy and reproducibility of repeat injections of the certified reference material BCR 579 spiked with 2.0 ng L^{-1} of CH_3Hg versus an external calibration with standards made in ultra-pure water and a matrix matched calibration with standards made in filtered undiluted seawater

Sample	Hg^{2+} spike recovery (%)	CH_3Hg spike recovery (%)
10 ng L^{-1} spike ($n = 3$) ± 1S.D.	108 ± 4	84 ± 3
Sample	**Hg^{2+} (ng L^{-1})**	**CH_3Hg (ng L^{-1})**
BCR 579[a] ($n = 5$) versus an external calibration	2.21[b] ± 0.55	1.85[c] ± 0.23
BCR 579[a] ($n = 5$) versus a matrix matched calibration	1.86[b] ± 0.34	2.1[c] ± 0.14

[a] BCR 579 coastal seawater reference material certified value 1.85 ± 0.2 ng L^{-1}.
[b] (Result ± 1S.D.).
[c] CH_3Hg spiked at 2.0 ng L^{-1} result ± S.D.

Table 5 – Analysis of the particulate and dissolved phases of water samples from Venetian canals for mercury species

Sample number	Volume filtered (mL)	Mean concentration in extract (ng L^{-1}) (S.D.) $n = 3$		Mean concentration in the particulate phase (ng L^{-1})		Mean concentration in the dissolved phase (ng L^{-1}) (S.D.) $n = 2$	
		Hg^{2+}	CH_3 Hg^+	Hg^{2+}	CH_3Hg^+	Hg^{2+}	CH_3Hg^+
1	510	N.D.	48.0 (0.5)	N.D.	0.29	0.24 (0.07)	0.06 (0.02)
2	500	N.D.	66.3 (2.1)	N.D.	0.40	0.54 (0.05)	0.13 (0.01)
3	500	N.D.	72.1 (0.1)	N.D.	0.46	0.38 (0.07)	0.07 (0.02)
Filter blank	500	3.2 (1.2)	4.9 (2.2)				

over estimates the Hg^{2+} content of the spiked samples and under estimates the CH_3Hg content of the spiked samples, which is reflected in the results for the spiked BCR 579 reference material. This suggests that there is a matrix effect on the pre-concentration phase that needs to be corrected for. The standard additions calibration (matrix matched calibration), when used to quantitate the Hg^{2+} and CH_3Hg levels in the reference material spiked with CH_3Hg give excellent agreement with the certified and spike values, respectively, showing that matrix matching the standards with seawater adequately corrects the matrix effects previously identified.

3.4. *Sample analysis*

To see if the method was suitable for monitoring mercury levels in the Venice lagoon three samples of surface water from canals close to the University were analysed for inorganic and CH_3Hg. The samples were collected in clean glass bottles and were transported to the laboratory for immediate analysis. Aliquots of the samples (20 mL) were filtered and injected onto the pre-concentration column; the column was then washed with 500 μL of ultra-pure water to remove the seawater matrix.

To investigate the concentration of CH_3Hg associated with the particulate phase, approximately 500 mL of the 3 separate unfiltered, unacidified samples of Venice canal water were filtered using the filters specified above. These samples had been left deliberately unacidified to avoid disturbing the equilibrium between the particulate and dissolved phases. The filters were transferred into 15 mL amber glass vials and the CH_3Hg present was extracted from the particulate matter immobilized on the filter using 6 mL of an extraction solution of 7% (v/v) HCl and 1% (v/v) 2-mercaptoethanol with an ultrasonic bath set to 60 °C with a sonication extraction time of 30 min. The extract was filtered using 0.45 μm syringe filters and diluted 1:1 with ultra-pure water. A calibration blank and the mixed mercury species standards (calibration range 0–500 ng L^{-1} of inorganic mercury and CH_3Hg) were made up in the diluted matrix (3.5% (v/v) HCl and 0.5% (v/v) 2-mercaptoethanol) in acid washed 25 mL amber glass bottles and were found to be stable for a week. As samples containing 2-mercaptoethanol cannot be pre-concentrated using the micro-column method described above, a 100 μL PEEK sample loop was fitted to the HPLC injection valve for sample introduction of the sample extracts. The results are summarised in Table 5, and show that after an effective pre-concentration of the particulate phase on a filter, the CH_3Hg concentration is easily quantifiable with a good precision (<1% R.S.D.). Our results for the particulate phase range from 0.29 to 0.46 ng L^{-1} which although from a small number of samples are similar to those found by Bloom et al. [17] who found values ranging from 0.05 to 0.27 ng L^{-1}, the results of this author for CH_3Hg in the dissolved phase (filtered with 0.45 μm filters) are similar to ours with values ranging from 0.02 to 0.10 ng L^{-1}. The results for the mercury levels in the dissolved phase show that the method is sensitive enough to detect inorganic and methyl mercury levels in the Venice lagoon, although methyl mercury levels are close to our detection limits so these analyses may need to be carried out with larger volumes during monitoring campaigns. This will require the use of higher pressure fittings for the peristaltic pump, or the use of a syringe pump or HPLC pump to load the pre-concentration columns off-line before use.

4. Conclusions

Methods for the determination of inorganic and CH_3Hg in the dissolved (filtered before analysis) phase of natural waters and for the determination of CH_3Hg in the particulate phase of natural waters has been developed. The use of a micro-column in place of the sample loop in the injection valve allowed the rapid and reproducible pre-concentration of dissolved mercury species and the removal of possible matrix interferences present in seawater (such as Na and Ca) prior to their determination. This method has been successfully applied to samples from the Venetian lagoon, an important environment at the northern end of the Adriatic Sea. The results found for Hg^{2+} agree well with a certified reference material, BCR 579, coastal seawater, certified for mercury and the results for dissolved levels of CH_3Hg are similar to those reported in the literature for this environment. This method is not suitable for the direct determination of CH_3Hg in unfiltered samples, so the filtrate was collected for samples of up to 500 mL. Extraction of this filtrate enabled the determination of CH_3Hg associated with the particulate phase present at levels below 1 ng L^{-1}. Although this methodology does not reach the detection limits of GC–ICP-MS with SPME or purge and trap pre-concentration that have been used for the determination of mercury species in pristine environments; it is suitable for the determination of mercury species in large numbers of samples from polluted aquatic environments, as the time required for pre-concentration and matrix removal is less than a minute per replicate.

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